

**The Study of Biomass Yield and Macromolecular Content of Microalgae Change as  
a Function of the Physiological State and Nutrient Supply Conditions**

By

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## ABSTRACT

Recently, as the crisis of conventional fossil fuel and air pollution has become more and more serious, microalgae-based biofuel is treated as one of the most promising new energy sources to solve this issue. By knowing the relationship of algal biomass and macromolecular content, nutrient composition and physiological states, the optimal growth condition and maximum biomass and biofuel productivity can be achieved. The aim of this study was to determine how the biomass and macromolecular content varies with growth phases and cultivation parameters including pH, N:P, medium type and algal species.

This experiment was based on 144 L batch-cultured green microalgae, *C. kessleri* and *A. falcatus*. pH 6.5 was more favored by *C. kessleri* than pH 8.5 since pH 6.5 could allow more biologically-available  $\text{CO}_2$  and  $\text{HCO}_3^-$  dissolved in the water as carbon resource for algae than alkaline pH. There was a decline in biomass concentration and protein content and an increase in lipid content for both species in N-limited condition, compared with N-sufficient condition. In the stationary phase, the highest lipid content of 54% of dry biomass was achieved in *C. kessleri* due to a severe deficiency of nitrogen in WC medium. *C. kessleri* removed 71% of phosphorus from WC medium with N:P = 2:1 as compared to 39% of phosphorus removal from WC medium with N:P = 50:1, indicating an excessive uptake of phosphorus of *C. kessleri* in N-limited condition. Meanwhile, a luxury consumption of nitrogen was found in N-sufficient condition since the N:P removal ratio from the medium was 38.5:1, which was higher than Redfield Ratio of 16:1. This excessive storage of nutrient caused a significant increase in ash content in the stationary phase in all conditions. *C. kessleri* performed better in biomass accumulation and lipid yield than *A. falcatus* in WC medium. *C. kessleri* showed 1.3 times higher maximum biomass concentration but about 4 times longer cultivation time in Bristol medium compared to WC medium, which was attributed to the much higher nitrogen and phosphorus concentration in Bristol medium. DOC above 5.5 mg-C/L might have a negative effect on the growth of *C. kessleri* because of its shading effect and combination of nutrient compounds.

Overall, the maximum biomass and lipid yield was found in *C. kessleri* fed with N:P = 2:1 Bristol medium at pH 6.5. The findings in this study can be used as guidance for optimizing cultivation conditions to harvest maximum biomass and lipids in future field-scale experiments.

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# 1 INTRODUCTION

High global energy demand of fossil fuels has triggered a contemporary energy crisis. Meanwhile, excessive consumption of fossil fuels in both developed and growing economies elevated the concentration of greenhouse gas, such as carbon dioxide ( $\text{CO}_2$ ), nitrous oxide ( $\text{N}_2\text{O}$ ), sulfur dioxide ( $\text{SO}_2$ ) and methane ( $\text{CH}_4$ ) and made the environment even worse in the past few decades. These concerns has driven an interest in developing alternative sustainable energy sources (Lv et al., 2010).

Biodiesel, derived from biomass, has been widely considered one of the most promising renewable fuels and has significant possibilities to replace fossil fuels in decades. It can contribute to fulfilling global energy demand in environmentally friendly manner by providing greener heat, power and transportation fuels with much less greenhouse emission than petroleum and coals (Phukan et al., 2011).

However, conventional biofuels usually derive from valuable food crops and oil seeds. The production process reduces available food resources for human beings. As an alternative to the "food versus fuel" challenge, microalgae can be cultivated as a renewable feedstock that has higher biomass and lipid production than traditional feedstocks. Furthermore, microalgae can grow in municipal wastewater and uptake the nutrients such as nitrogen and phosphorus from the wastewater to produce biomass and lipids. Therefore, little or no artificial fertilizer needs to be added to facilitate the growth

of microalgae. On the other hand, the growth of microalgae helps to remove nitrogen and phosphorus in the wastewater and this process can greatly reduce the cost of wastewater treatment.

Most of previous studies on biofuel production were mainly focused on how to identify the optimal cultivation conditions for the maximum biomass yield and lipid production (Chisti et al., 2011). The influencing factors include the algae species, nutrient composition and cultivation patterns. In addition, it is reported that the N:P ratio in the medium has a strong impact on the macromolecular content of microalgae (Lai et al., 2011). In order to comprehensively understand the effects of different cultivation conditions on algal growth and biochemical components, more research needs to be conducted.

In this study, the variation of algal growth and macromolecular content as a function of physiological state with different cultivation parameters will be investigated in order to find out the relationship between algal biochemical composition, nutrient conditions and physiological states. These cultivation parameters include algal species, pH, N:P and medium type, all of which are considered as crucial factors to algal growth. The study will determine the optimal cultivation conditions to achieve maximum biomass and lipid yield. Furthermore, it will also provide a better understanding of the process of algal growth and lipid production.

## **2 LITERATURE REVIEW**

### **2.1 Microalgae**

Microalgae are unicellular or multicellular microorganisms. In general the maximum diameter of a single cell is only 50µm. They can live in a variety of environmental conditions including saline or alkaline habitats, cold regions, hot springs and arid soils, when given sunlight and basic nutrients. Mostly microalgae are free-living organisms except for some species living as parasites or in symbiotic relationship with other organisms (Nabors et al., 2004). Microalgae can be prokaryotes or eukaryotes depending on their cell structure. Prokaryotic microalgae are often referred to as cyanobacteria (cyanophyceae). Microalgae are sunlight-driven factories that can convert the carbon dioxide absorbed from atmosphere into cellular macromolecules like proteins, pigments, lipids and carbohydrates (Metting, 1996; Spolaore et al., 2006).

### **2.2 Production of Microalgae-based Biodiesel**

There is a rapidly growing interest in the potential of microalgae as feedstocks for the second generation of biofuels. According to the special characteristics of cell structures and metabolism ways, microalgae have many advantages as biodiesel raw materials compared with other materials like terrestrial crops. Since microalgae possess small and simple cellular structures, the general biomass doubling time in the log phase can be as short as 3.5 hours (Chisti, 2007). Microalgae are capable of

metabolizing nutrients in the water and carbon dioxide in the atmosphere to produce high content of lipid, which is very common in the range of 20-50% of dry biomass weight (shown in **Table 2-1**). High lipid content, high biomass productivity and high growth density allow microalgae consuming much less land for cultivation to meet the global energy demand (shown in Table 2). Except that, **Table 2-2** also shows that only microalgae-based biodiesel is likely to become the fuel that can replace fossil fuels in several decades. The biomass and oil can be harvested nearly all-year-round, providing a reliable and continuous supply of biodiesel. Moreover, the cultivation of microalgae can be conducted on the surface of the ocean so that it will reduce the competition with crops for available-arable land (Schenk et al., 2008). Great efficiency of light capture and biochemical conversion make microalgae was able to grow by using CO<sub>2</sub> from power plants and nutrients from wastewater so that it can result in reduction of CO<sub>2</sub> emission, fertilizer usage and waste nutrients concentration (Mallick, 2002; Schenk et al., 2008).

**Table 2-1. Lipid content of some microalgae (% dry weight) (Gouveia et al., 2009)**

Species	Lipids
<i>Scenedesmus obliquus</i>	11–22/35–55
<i>Scenedesmus dimorphus</i>	6–7/16–40
<i>Chlorella vulgaris</i>	14–40/56
<i>Chlorella emersonii</i>	63
<i>Chlorella protothecoides</i>	23/55
<i>Chlorella sorokiana</i>	22
<i>Chlorella minutissima</i>	57
<i>Dunaliella bioculata</i>	8
<i>Dunaliella salina</i>	14–20
<i>Neochloris oleoabundans</i>	35–65
<i>Spirulina maxima</i>	4–9

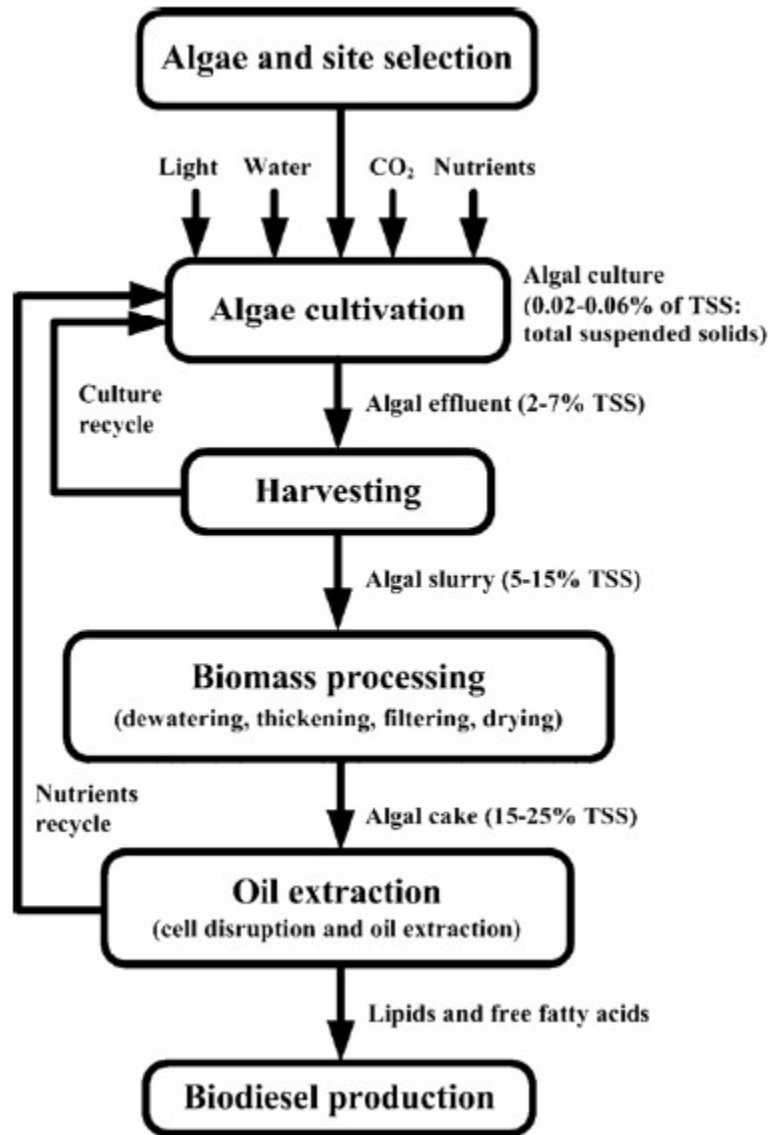
**Table 2-2. Comparison of some sources of biodiesel (Chisti, 2007)**

Crop	Oil yield (L/ha)	Land area needed (M ha) <sup>a</sup>	Percent of existing US cropping area <sup>a</sup>
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae <sup>b</sup>	136,900	2	1.1
Microalgae <sup>c</sup>	58,700	4.5	2.5

### 2.3 Advantages of Microalgae-based Biodiesel

Microalgae-based biodiesel is made from a series of processes (shown in **Figure 2-1**). Firstly algal culture were cultivated in the water and fed with essential nutrient

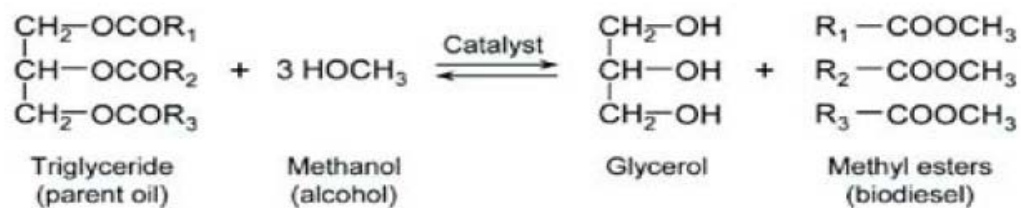
including CO<sub>2</sub>, light, nitrogen and phosphorus. Then the algal biomass is harvested and dewatered for lipid extraction. Finally the lipid is extracted with chloroform and methanol and later converted to biodiesel through transesterification process (Mata et al., 2010).



**Figure 2-1. Process of microalgae biodiesel production (Mata et al., 2010)**

Transesterification (see **Figure 2-2**) is a catalyzed (acid or base) process in which

TAGs (Triglyceride, a type of lipid) react with methanol or ethanol and the products are glycerol and fatty acid methyl/ethyl ester (FAME), which are the main molecules in biodiesel (Greenwell et al., 2010). Theoretically, transesterification needs 3 mol of alcohol for 1 mol of TAG to yield 1 mol of glycerol and 3 mol of methyl esters (Fukuda et al., 2001). This process is an equilibrium reaction. In order to make the yield of methyl esters exceed 98% on a weight basis, industrial processes use 6 mol of methanol for 1 mol of TAG to drive the reaction to the right side (Fukuda et al., 2001).



**Figure 2-2. Transesterification of lipid to biodiesel (Chisti, 2007)**

## 2.4 Algae Species

Depending on the pigmentation, specific food reserves and the characteristics of their cell wall structures (Tiamiyu, 2011), algae are sorted into different phyla. Due to the capability of storing energetic macromolecules like carbohydrates and lipids, these microalgae can be used in producing biofuel and categorized as *Chlorophyta* (Green algae), *Bacillariophyta* (Diatomas), *Chrysophyta* (Golden-brown algae), *Rhodophyta* (Red algae), *Cyanobacteria* (Blue-green algae) and *Phaeophyta* (Brown algae) (Tiamiyu, 2011).



In the research presented herein, *C. kessleri* and *A. falcatus* are chosen. *C. kessleri* has shown a good endurance for various conditions. They can adapt themselves in various habitats like damp soil, open ponds and even the surface of tree trunks (Mata et al., 2010). This characteristic makes them very readily cultivated worldwide. The other characteristic for *C. kessleri* is their high lipid content and fast growth rate. **Table 2-3** shows *C. kessleri* obtained lipid content up to 48%~58% of dry biomass weight. *C. kessleri* seems to be a good option for maximizing biodiesel production when cultivated in low nitrogen medium (Illman et al., 2000). The biodiesel derived from *C. vulgaris* species in N-stress environment has been successfully applied to an unmodified single cylinder diesel engine (Scragg et al., 2003). High quality biodiesel was achieved from *Chlorella* cultivated in heterotrophic conditions (Xu et al., 2006).

*A. falcatus* can survive either in marine or freshwater systems. Like terrestrial plants, *A. falcatus* has chlorophyll a and b and store starch in plastids as food reserves, which can be altered under N-deficiency to accumulate lipids (Round et al., 1990). By investigating the effects of NaCl concentration on the growth and lipid content of *A. falcatus*, a recent study found that the highest total lipid content can reach 56.1% of dry cell weight in Bold's Basal medium with 0.17 M NaCl (Kalita et al., 2011). If this high lipid content can be associated with optimal biomass production strategies, *A. falcatus* will be very promising for producing biofuel.

**Table 2-3. Lipid content and productivities of different microalgae species (Mata et al., 2010)**

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)
<i>Ankistrodesmus</i> sp.	24.0–31.0	–	–
<i>Botryococcus braunii</i>	25.0–75.0	–	0.02
<i>Chaetoceros muelleri</i>	33.6	21.8	0.07
<i>Chaetoceros calcitrans</i>	14.6–16.4/39.8	17.6	0.04
<i>Chlorella emersonii</i>	25.0–63.0	10.3–50.0	0.036–0.041
<i>Chlorella protothecoides</i>	14.6–57.8	1214	2.00–7.70
<i>Chlorella sorokiniana</i>	19.0–22.0	44.7	0.23–1.47
<i>Chlorella vulgaris</i>	5.0–58.0	11.2–40.0	0.02–0.20
<i>Chlorella</i> sp.	10.0–48.0	42.1	0.02–2.5
<i>Chlorella pyrenoidosa</i>	2.0	–	2.90–3.64
<i>Chlorella</i>	18.0–57.0	18.7	–
<i>Chlorococcum</i> sp.	19.3	53.7	0.28
<i>Cryptocodinium cohnii</i>	20.0–51.1	–	10
<i>Dunaliella salina</i>	6.0–25.0	116.0	0.22–0.34
<i>Dunaliella primolecta</i>	23.1	–	0.09
<i>Dunaliella tertiolecta</i>	16.7–71.0	–	0.12
<i>Dunaliella</i> sp.	17.5–67.0	33.5	–
<i>Ellipsoidion</i> sp.	27.4	47.3	0.17
<i>Euglena gracilis</i>	14.0–20.0	–	7.70
<i>Haematococcus pluvialis</i>	25.0	–	0.05–0.06
<i>Isochrysis galbana</i>	7.0–40.0	–	0.32–1.60
<i>Isochrysis</i> sp.	7.1–33	37.8	0.08–0.17
<i>Monodus subterraneus</i>	16.0	30.4	0.19
<i>Monallanthus salina</i>	20.0–22.0	–	0.08
<i>Nannochloris</i> sp.	20.0–56.0	60.9–76.5	0.17–0.51
<i>Nannochloropsis oculata</i>	22.7–29.7	84.0–142.0	0.37–0.48
<i>Nannochloropsis</i> sp.	12.0–53.0	37.6–90.0	0.17–1.43
<i>Neochloris oleoabundans</i>	29.0–65.0	90.0–134.0	–
<i>Nitzschia</i> sp.	16.0–47.0	–	–
<i>Oocystis pusilla</i>	10.5	–	–
<i>Pavlova salina</i>	30.9	49.4	0.16
<i>Pavlova lutheri</i>	35.5	40.2	0.14
<i>Phaeodactylum tricornutum</i>	18.0–57.0	44.8	0.003–1.9
<i>Porphyridium cruentum</i>	9.0–18.8/60.7	34.8	0.36–1.50
<i>Scenedesmus obliquus</i>	11.0–55.0	–	0.004–0.74
<i>Scenedesmus quadricauda</i>	1.9–18.4	35.1	0.19
<i>Scenedesmus</i> sp.	19.6–21.1	40.8–53.9	0.03–0.26
<i>Skeletonema</i> sp.	13.3–31.8	27.3	0.09
<i>Skeletonema costatum</i>	13.5–51.3	17.4	0.08
<i>Spirulina platensis</i>	4.0–16.6	–	0.06–4.3
<i>Spirulina maxima</i>	4.0–9.0	–	0.21–0.25
<i>Thalassiosira pseudonana</i>	20.6	17.4	0.08
<i>Tetraselmis suecica</i>	8.5–23.0	27.0–36.4	0.12–0.32
<i>Tetraselmis</i> sp.	12.6–14.7	43.4	0.30

## 2.5 Influencing Factors on Algal Growth and Lipid Yield

### 2.5.1 pH and Carbon Species

Different species have their own optimal range of conditions in which they thrive.

Neutral pH is favored by most freshwater green algae. Maintaining an optimum pH is

essential for algae cultivation. In fact, constant pH and sufficient buffering capacity of medium also play a very crucial role to algal growth. Only by combination of these two factors, could the maximum biomass and lipid productivity be obtained. Conventionally, a freshwater system is considered as a weak pH buffering system. Whereas, a couple of studies have shown that significant fluctuation of pH occurs in marine water as well (Park et al., 1958; Pegler et al., 1988). Other than high pH fluctuation, high pH also has a negative effect on algal abundance and productivity due to a reduction of utilization efficiency of carbon source (Talling, 1976). The tolerance of high pH and low concentration of CO<sub>2</sub> might be a reason for species distribution and succession (Goldman, 1973) in both marine and fresh water.

pH can influence metabolism of microalgae by altering the distribution of three forms of dissolved inorganic carbon and changing the availability of some essential metal ions like calcium in the water (Chen et al., 1994). Dissolved inorganic C-species in the medium include H<sub>2</sub>CO<sub>3</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup>, and algae require HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> to facilitate photosynthesis. Especially when algae live in the environment with lower alkalinity, C-species become more important for their living as not only a carbon resource but also a pH stabilizer.

The main sources of inorganic carbon are the CO<sub>2</sub> in the atmosphere and carbonate dissolved from rocks. Both of these natural processes may take long time to accumulate sufficient carbon nutrient and alkalinity in the aqueous system, since neither the diffusion

rate from air phase to water phase nor dissolution rate from solid phase to water phase can compete with CO<sub>2</sub> assimilation rate in algal cells. Therefore, CO<sub>2</sub> is often a constraint in this kind of system for high yields of algal biomass(Becker, 1994; Brewer et al., 1976).CO<sub>2</sub> has to be enriched continuously to obtain the high growth rate and productivity of microalgae in the water (Williams et al., 2010). It was reported that when constant concentration of CO<sub>2</sub>is bubbled, *C. vulgaris* can fix CO<sub>2</sub> from lower than 25 mg/L/h to 52 mg/L/h when CO<sub>2</sub> increase from 5% to 20%(Yun et al., 1996). Similar results were also found in other algae species (Brune et al., 2009; Chiu et al., 2009). Considering cost and environment, microalgae can be fed with waste gas from industrial processes (i.e., diesel engines, cement plants or fermentation reactors) as an inorganic carbon source (Tiamiyu, 2011).A previous study reported that *Scenedesmusobliquus* and *C. kessleri* cultures, isolated from a treatment pond in a coal-fired thermoelectric power plant in Brazil, showed a capacity of bio-fixation of CO<sub>2</sub>(de Moraes et al., 2007). Enhanced addition of CO<sub>2</sub>fine bubbles can not only accelerate photosynthesis and promote primary production but also increase lipid content. In an investigation on the effect of adding CO<sub>2</sub> on lipid accumulation in *Nannochloropsisoculata*, it was shown that adding 2% of CO<sub>2</sub>canincrease the lipid content from 30.8% of dry biomass in the log phase to 50.4% in the stationary phase(Chiu et al., 2009). Recently, it was suggested that ~1% concentration of CO<sub>2</sub> input, proper nitrate concentration and efficient irradiance operation can maximize lipid productivity (Lv et al., 2010).

### **2.5.2 Nitrogen**

Nitrogen is one of the most fundamental and significant nutrients for microalgal growth. It is typically in forms of nitrate, nitrite, ammonia or urea. Nitrogen is a vital component/element for composition, formation and functionality of protein and DNA in microalgal cells (Simon, 1971). The average nitrogen requirement for most green algae is between 5-50 mM(Becker, 1994).

When nitrogen supply is sufficient, the dominant biochemical component in most microalgae is protein other than lipids. When it comes to a nitrogen-limited condition, some proteins will be vigorously degraded to provide nitrogen resources for maintaining the normal metabolism. Meanwhile the carbon flow, fixed by algae in photosynthesis, will contribute to synthesizing protein, carbohydrate and lipids. Without sufficient stock materials to produce protein, some fixed carbon will shift to synthesize lipids other than protein. In Weissman's study(Weissman, 1984), nitrogen-stress condition caused a 10% increase of lipid content in *Botryococcus*. Illman also reported that a reduction of nitrogen in the medium can increase the lipid content up to 63%, 56% and 40% of dry cell weight for all three investigated *Chlorella* strains: *C. emersonii*, *C. minutissima* and *C. vulgaris* respectively(Illman et al., 2000).

### **2.5.3 Phosphorous**

Phosphorous is an essential element for cellular metabolic processes in algal cells,

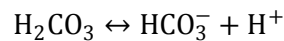
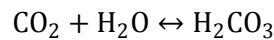
especially cell growth, biosynthesis of nucleic acid and energy transfer(Becker, 1994). Phosphorous concentration usually is the growth rate limiting factor in natural aquatic environment. Phosphorous exists in natural water as either orthophosphate or organic phosphate. Inorganic phosphate can exist in cells as polyphosphate, storing specific polyphosphate granules that emerge in cells when phosphorous is adequate but disappear in phosphorous-depleted growth conditions (Tiamiyu, 2011).

Phosphorous in forms of  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  can be assimilated by microalgae, and the assimilation rates depend on the phosphorous concentration in the medium and microalgae species. Phosphorus stress has a similar effect on macromolecular composition change in microalgal cells as nitrogen stress does, including decrease in protein and chlorophyll levels and increase in lipid and possible carbohydrate fractions(Becker, 1994). Distinctively, the degradation of phycobilisome (a light-harvesting complex functioning in photosynthesis process) in algal cell may be mostly in P-deficient conditions compared with N-limited environments (Tiamiyu, 2011). Some recent studies have observed the limitation of either nitrogen or phosphorus in the medium has a negative influence on the uptake of other nutrients (Lai et al., 2011). Additionally the removal rate of N:P in N-deficient medium was found greater than biomass Redfield ratio (16:1), indicating luxury P consumption in cells (Hou et al., 2007). These phytoplankton species can store excess phosphorus for use in P stress conditions. This ability of luxury P consumption may contribute to the survival of algae in P-limited environments like algae blooms since

P is most likely to be exhausted first compared with nitrogen when a bloom occurs(Lai et al., 2011).

#### 2.5.4 Alkalinity and DOC

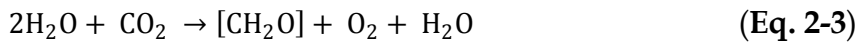
A buffering system based on the carbonate system(see **Eq. 2-1**) and other weak bases and acids can prevent large changes in pH when an acid or base is added to the water. This buffering capacity is referred to as total alkalinity (TA). TA can be demonstrated in a simplified equation (**Eq. 2-2**) since the concentration of other negatively charged molecules are nearly always substantially less than 1% compared with carbonate and bicarbonate in fresh water(Stumm, 1970).Sufficient alkalinity is crucial inmaintaining aconstant pH in the water, thereby guaranteeing a constant intercellular pH of phytoplankton.



$$\text{Total Alkalinity} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{OH}^-] + [\text{H}^+](\text{Eq.2-2})$$

Adding CO<sub>2</sub> will increase DIC (dissolved inorganic carbon). One molecule of HCO<sub>3</sub><sup>-</sup> is producedwith an associated release of one H<sup>+</sup>, canceling any net increase in alkalinity. Therefore, there are typically no net changes in alkalinity with CO<sub>2</sub> input and removal in the water during CO<sub>2</sub> compensation from atmosphere (Stumm, 1970).However, a

decrease in pond water alkalinity is suspected to occur when  $\text{CO}_2$  and  $\text{HCO}_3^-$  is removed by algae in photosynthesis (**Eq. 2-3**) , and this can result in an increase in pH in the system.

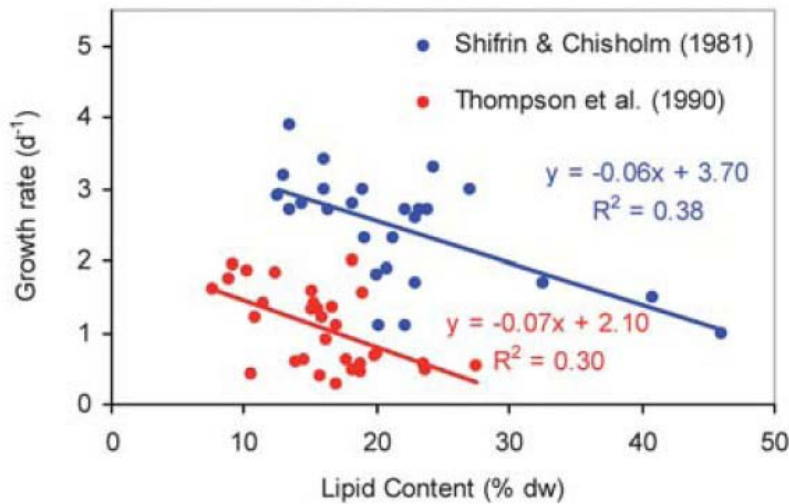


Commonly there are two way for algae to release DOC into water: (1) an equilibrium driving force in and out of cytoplasmic membrane (Type I )and (2) degradation of dead cells and extracellular organic matter (Type II ) (Watt, 1966). Most DOC, released in the water by microalgae in the exponential phase, was Type I DOC. This type of DOC mainly consists of intermediate products of metabolism, including glycolic and amino acids. However, with the DOC increasing in the water from exponential to log phase, the release of this type of DOC will decrease. Based on the Watt's observation, high dilution of medium can efficiently enhance the productivity of Type I DOC. DOC of Type II is featured with higher molar weight than Type I since it is dominated by larger macromolecules like polysaccharides(Nguyen et al., 2005; Watt, 1966). Although DOC is biologically available to microalgae(Nguyen et al., 2005), anegativeeffect of DOC on biomass accumulation was concluded by observing a reduction of primary production with increasing DOC from 5 mg-C/L to 17 mg-C/L (Carpenter et al., 1998).



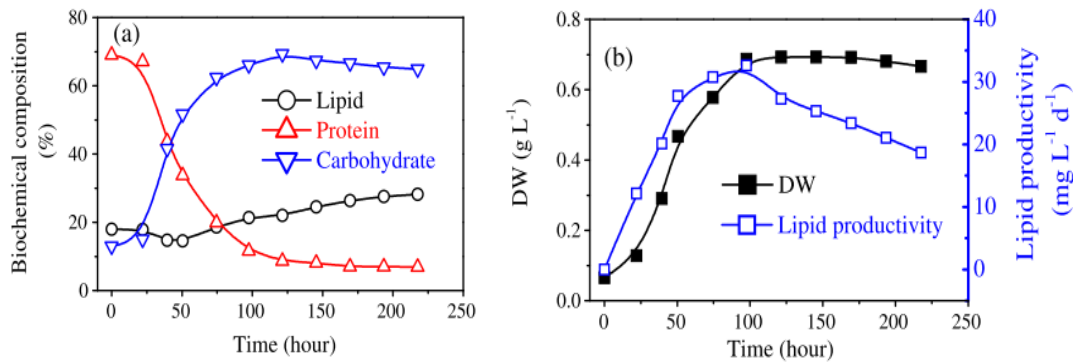
### **2.5.5 Relationship between Different Physiological States and Macromolecular Content of Microalgae**

Different physiological phases have significant effects on microalgal biochemical composition such as lipid, protein and carbohydrate. Shifrin reported the relationship of growth rate and lipid content by studying 15 strains of freshwater and 11 strains of marine eukaryotic algae (Shifrin et al., 1981). The data were taken in the exponential phase. Same topic was also studied by investigating 8 eukaryotic marine algae at stationary phase (Thompson et al., 1990). The results of these two studies were summarized and analyzed by Williams and Laurens (Williams et al., 2010), shown in **Figure 2-3**. It indicates that an increase in lipid content from 15% -30% can lead 50% or more decrease of growth rate. Promoting lipid productivity by only driving the algae to nutrient deficiency would cause other problems. It not only prolongs culturing time but also reduces the maximum biomass concentration. Williams and Laurens suggested that the limited potential in lipid increase at the present is 35% of dry weight.



**Figure 2-3.** Variation of growth rate with lipid content of some marine and freshwater eukaryotic algae;  $p < 0.006$  in Shifrin and Chisholm's data;  $p < 0.0014$  in Thompson's data (Williams et al., 2010)

As shown in **Figure 2-4**, it has been suggested that the maximum lipid production occurs at the early stationary phase not exponential phase when the specific growth rate is highest (Lv et al., 2010). As growth rate slows down to stationary state along with cultivation time in the batch culture, algal cellular protein concentration drops, and carbohydrate content significantly increases (Lv et al., 2010). In this batch culture, the decline of protein content results from N-limitation since nitrogen is a major component of protein structure. This nutrient-limited condition also suppresses algal cells division but promotes the lipid productivity.



**Figure 2-4.** Variation of biochemical compositions, dry weight (DW) and lipid productivity of batch culture of *C. vulgaris* fed with 1.0% CO<sub>2</sub>, N:P =1:7, 1.0 mM KNO<sub>3</sub>, continuous light (60  $\mu$ mol photos/m<sup>2</sup>s) and temperature 25°C (Lv et al., 2010)

### 2.5.6 Gaps between Literature and Current Research

There is a complex relationship among species selection, cultivation conditions, and biomass and lipid production. A large proportion of the studies related to this topic utilize small-scale algal culture below 20 L. By studying and comparing the variation of algal growth and biochemical compositions (protein, carbohydrates and lipids) of two potential strains in different cultivation conditions (pH, N:P and medium type), this research was aimed to inform the optimal species, growth state and growth conditions to maximize algal biofuel production. A larger-scale experimental set-up (144L) was chosen to provide a reliable and replicate data.

## 3 RESEARCH QUESTIONS

How do the biomass yield and macromolecular content of microalgae change as a function of the physiological state and nutrient supply conditions?

## 4 METHODS

### 4.1 Strains and Media

Microalgae used for this study were *C. kessleri* (UTEX 2228) and *A. falcatus* (provided by Dr. Val Smith, University of Kansas). These two strains of green algae were selected due to their relatively high lipid productivity, ability to survive shear stress, high CO<sub>2</sub> sinking capacity, low nutrient requirement, fast growth rate and high photosynthesis efficiency.

Bristol medium (N:P=2:1 by moles) and WC medium (N:P=2:1 and N:P=50:1 by moles) were utilized to grow freshwater green microalgae. The recipe for each is shown in **Table 4-1** and **Table 4-2**. The nitrogen to phosphorus ratio is the molar ratio of nitrogen and phosphorus in the medium. The ratio can vary depending on the amount of inorganic nitrogen and phosphorus being added. On the other hand, the atomic ratio of carbon, nitrogen and phosphorus for general species of phytoplankton is described with Redfield ratio. Since the Redfield ratio is 106:16:1, an N:P ratio by moles of 50 represented a nitrogen-sufficient (phosphorus-limited) medium while N:P ratio of 2 represented a nitrogen-limited medium.

**Table 4-1. Recipe of Bristol medium with N:P = 2:1 (by moles)**

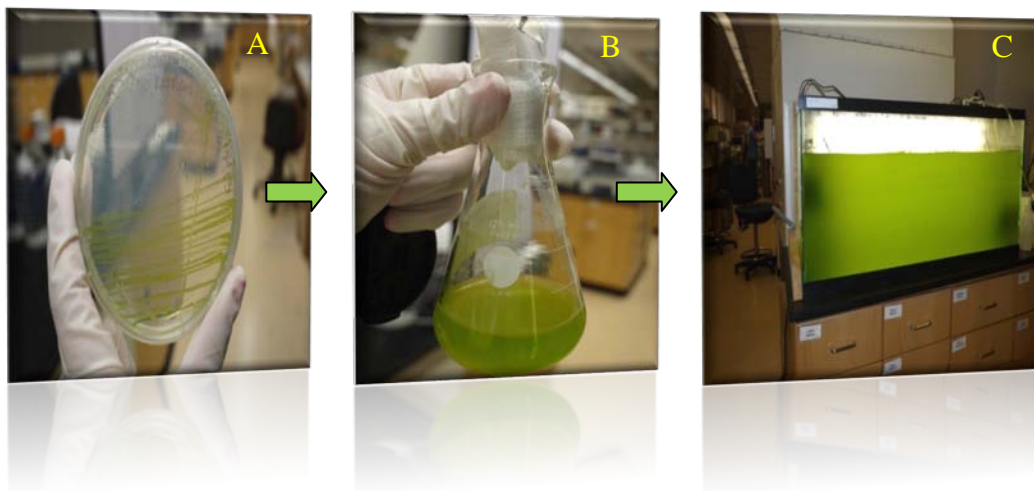
<b>Chemical Formula</b>	<b>Bristol Medium (N:P = 2:1) Concentration in Medium</b>	
	$\mu\text{M/L}$	mg/L
NaNO <sub>3</sub>	2940	249.9
KH <sub>2</sub> PO <sub>4</sub>	1290	175.4
K <sub>2</sub> HPO <sub>4</sub>	430	74.8
CaCl <sub>2</sub> ·2H <sub>2</sub> O	170	25.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O	300	73.8
NaCl	430	25.2

**Table 4-2. Recipe of WC medium with N:P = 2:1 and 50:1 (by moles)**

<b>Chemical Formula</b>	<b>WC Medium (N:P = 2:1) Concentration in Medium</b>		<b>WC Medium (N:P = 50:1) Concentration in Medium</b>	
	$\mu\text{M/L}$	mg/L	$\mu\text{M/L}$	mg/L
NaNO <sub>3</sub>	100	8.5	2500	212.5
K <sub>2</sub> HPO <sub>4</sub>	50	8.7	50	8.7
CaCl <sub>2</sub> ·2H <sub>2</sub> O	250	36.8	250	36.8
MgSO <sub>4</sub> ·7H <sub>2</sub> O	150	36.9	150	36.9
NaHCO <sub>3</sub>	150	12.6	150	12.6
Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O	100	28.4	100	28.4
H <sub>3</sub> BO <sub>3</sub>	390	24.2	390	24.2
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	11.7	4.4	11.7	4.4
FeCl <sub>3</sub> ·6H <sub>2</sub> O	11.7	3.2	11.7	3.2
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.010	0.003	0.010	0.003
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.077	0.022	0.077	0.022
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.042	0.010	0.042	0.010
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.910	0.474	0.910	0.474
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.026	0.006	0.026	0.006
Na <sub>3</sub> VO <sub>4</sub>	0.098	0.018	0.098	0.018

## 4.2 Algae Cultivation and Batch System Operation

This research involved three batch experiments, and each one consisted of a combination of two aquariums. The total volume of batch culture in a aquarium was 144 L. Each batch culture started with a 100 ml sub-culture inoculated from pure culture grown on solid alga plates, demonstrated in **Figure 4-1**. In one experiment, sub-culture cultivation and batch system setup for each aquarium were conducted at the same time to minimum errors. Thus, all physical conditions were considered identical for two tanks except for the variables interested in this study. **Table 4-3** showed the variables and essential parameters in three batch experiments. Section 4.2.1 and 4.2.2 described the processes of sub-culture and batch-culture incubation respectively for experiment 1. These processes were repeated in experiment 2 and 3 except that some batches utilized different species, medium, N:P ratio and pH.



**Figure 4-1.**Process of scaling up plate culture to 144 L batch culture: (A) pure algae culture grown on a agar plate; (B) 100 ml pure sub-culture in a 300 ml Erlenmeyer flask; (C) 144 L pure batch algal culture

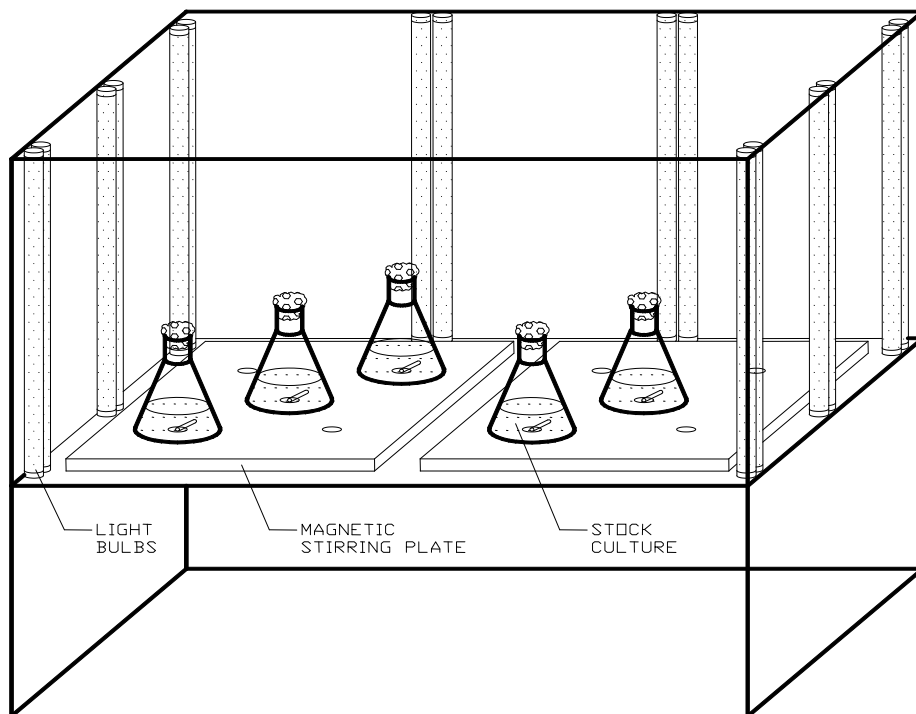
**Table 4-3. Summary of variables of two batches for all three aquarium experiments**

<b>Variables</b>	<b>Experiment 1</b>		<b>Experiment 2</b>		<b>Experiment 3</b>	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
<b>pH</b>	6.5	8.5	6.5	6.5	6.5	6.5
<b>Medium</b>	Bristol	Bristol	WC	WC	WC	WC
<b>N:P</b>	2:1	2:1	2:1	2:1	50:1	50:1
<b>Strains</b>	C <sup>1</sup>	C	C	A	C	A

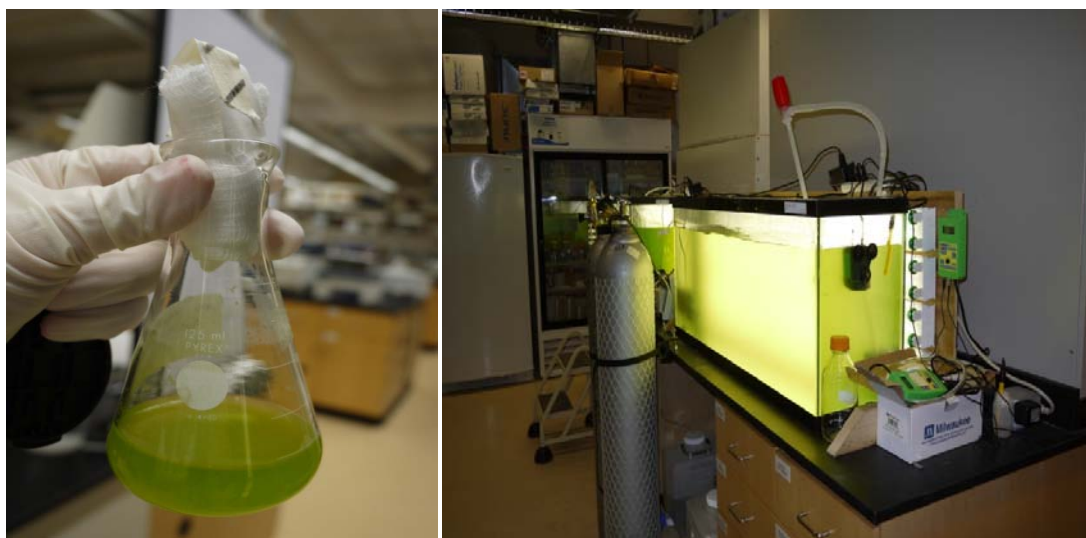
<sup>1</sup>C is *C. kessleri* and A is *A. falcatus*

#### 4.2.1 Pure Sub-culture Incubation

Batch cultures were prepared in two steps. *C. kessleri* and *A. falcatus* were initially incubated in two 300 ml Erlenmeyer flasks respectively. Erlenmeyer flasks were filled with 100 ml media, and a cheesecloth stopper was placed in each flask to prevent contamination but allow gas exchange. All flasks were autoclaved at 121 °C for 15 minutes for sterilization. Then pure cultures were introduced into different labeled flasks as stock cultures to provide the stock algal cells. All flask cultures were placed on two magnetic stirring plates under room temperature (20 – 25 °C). 16 florescent light bulbs (F34CW/RS/EW, Econ-o-watt Philips) were hung around the incubator as light source and were programmed with a timer (23ULD/3AO, Outdoor Timer, Woods) to provide a light cycle 14 hr light: 10 hr dark. For each experiment, a stock culture was inoculated for 5 days before introduced to larger scale batch reactors.



**Figure 4-2.**Schematic of algae sub-culture inoculation (100 ml liquid pure algae culture in 300 ml Erlenmeyer flask)



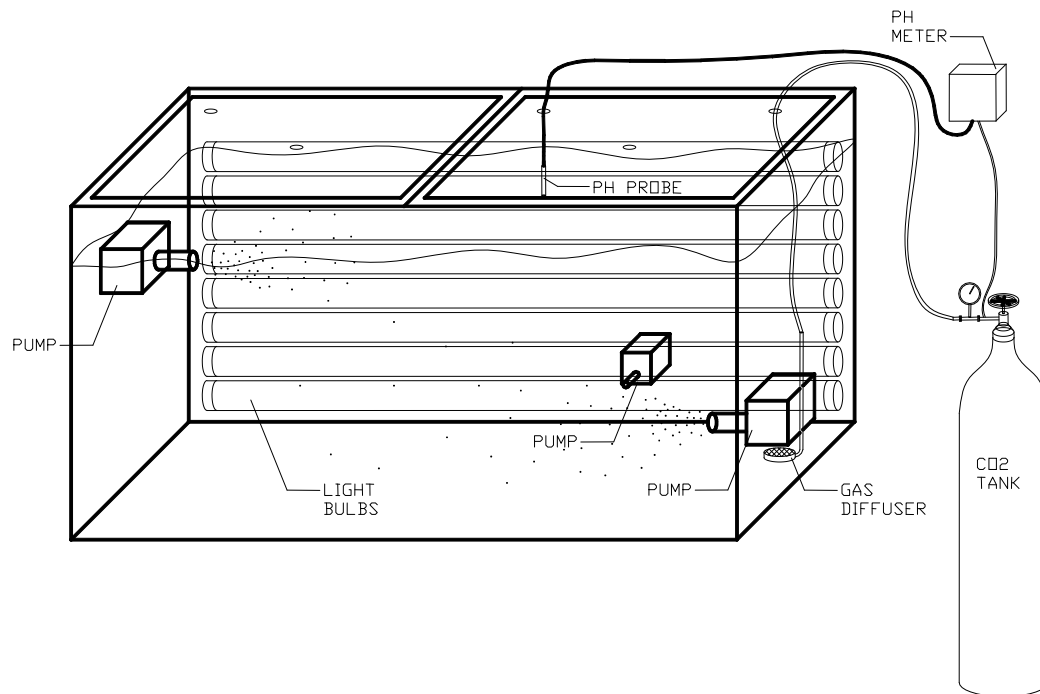
**Figure 4-3.**(A) 100 ml Sub-culture of *A. falcatus* and (B) 144 LA. *falcatus* batch culture in the apparent glass aquarium



#### 4.2.2 Batch Reactor Set-up

The second step was scaling up from 100 ml stock culture to 144 L batch cultures (see **Figure 4-3**). The configuration of batch system apparatus was shown in **Figure 4-4**. Two large transparent glass aquariums ( $L \times W \times H = 120\text{cm} \times 30\text{cm} \times 60\text{cm}$ ) were placed on a flat surface and sterilized with ethanol. 8 florescent light bulbs were installed vertically behind the aquariums to illuminate the algal culture. Same as the incubator, the batch reactor illumination system was operated with a 14 hrlight: 10 hr dark cycle. Each reactor included three submersible aquarium pumps, two large-volume pumps (Maxi-Jet Pump 3000, max 775 gal/h, Marineland) and one small-volume pump (Maxi-Jet Pump 900, max 245 gal/h, Marineland). One of the large pumps was installed at the water level (40 cm-height line) to facilitate mixing the culture and exchanging air. The other large pump and the small pump were placed near the bottom to prevent sedimentation and dead corners.  $\text{CO}_2$  was released from a gas diffuser. The head of the diffuser was taped with the large-volume pump near the bottom and the tail was connected to a  $\text{CO}_2$  cylinder with 1/8" tubing. In addition, the batch system had a smart pH meter (SMS122, pH meter, Milwaukee) to monitor pH. The supply of  $\text{CO}_2$  from  $\text{CO}_2$  cylinder was controlled to maintain the desired pH in the culture. Also 1 mol/L sodium hydroxide (NaOH) was prepared and placed beside the aquarium to bring up the pH if it was below preset level. After lighting, pumping and pH control were set up, deionized water was filled to the aquarium till water level reached 35 cm above bottom. Since stock solution of Bristol

medium was 100 times more concentrated than working solution, 1.44 L of Bristol stock solution was then transferred to the aquarium for making 144 L Bristol medium. For WC medium experiment, 0.144 L of WC stock solution was required for each batch culture for the reason that WC stock solution has 1000 times higher concentration than its working solution. Later, more deionized water was added in the aquarium later to bring the water level to 40 cm-height water line so that the total volume of medium in the aquarium was 144 L. A 10 ml sterile pipette was utilized to transfer 50 ml *C. kessleri* sub-culture by 5 times from Erlenmeyer flask to each batch reactor. Batch system was operated in well-mixing condition with constant pH.



**Figure 4-4. Schematic of aquarium experiment (144 L pure algae culture cultivated in 180 L glass aquarium, stirred by 3 submersible pumps and illuminated by 8 light bulbs. A smart pH meter was used to monitor pH in the medium and control CO<sub>2</sub> gas for maintaining a constant pH of batch culture)**

### 4.3 Algal Harvesting

Algal solution was pumped out manually from the aquarium to a glass container with a plastic pump. Enough volume of sample for all the tests and duplicate measurements was collected on each sampling event. For each batch experiment, sampling event was conducted for two to three times a week.

200 ml of algal solution was harvested in a 600 ml beaker for total suspended solids (TSS), volatile suspended solids (VSS), total alkalinity, dissolved organic carbon (DOC), pH and optical density (OD) tests. A magnetic stir bar was introduced into the glass container to achieve homogeneity for all samples. TSS/VSS, OD and total alkalinity, pH measurements were conducted immediately after culture harvested. More details for these tests are in Section 5.4.2, 4.5.4, 4.5.5, 4.5.10. Duplicate DOC samples were prepared and stored as described in Section 4.5.9.

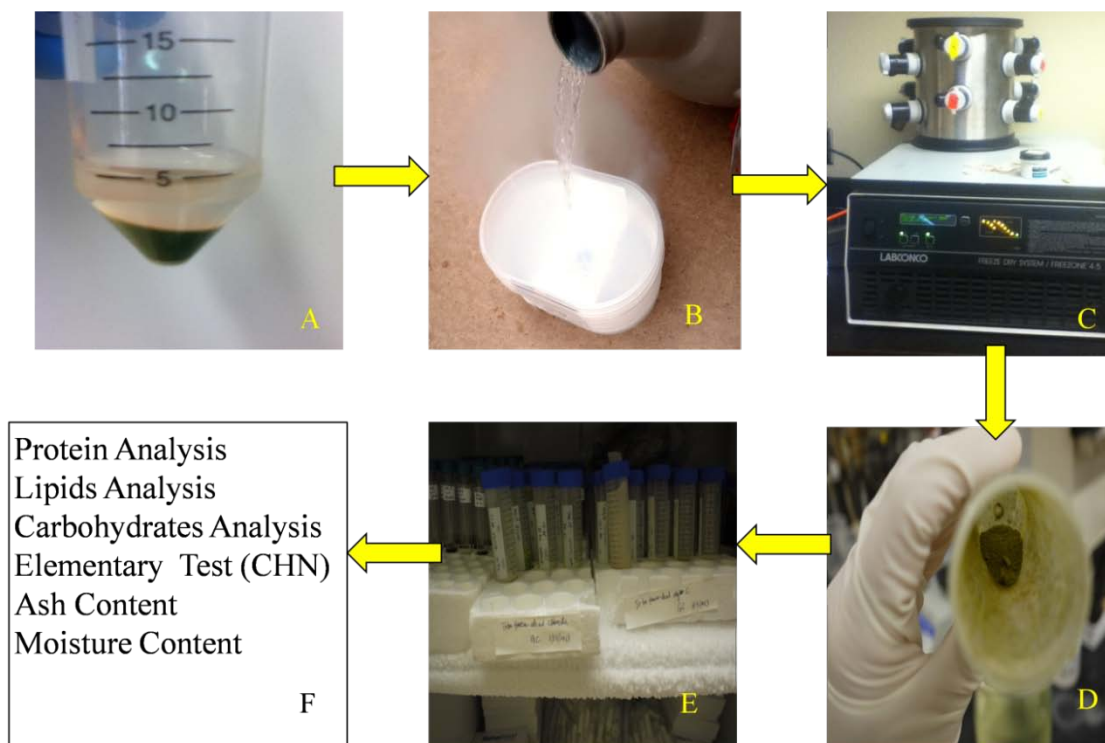
The other part of algal solution was collected for macromolecule, CHN and moisture and ash test. Previous study showed that VSS have a good correlation with OD, shown in **Figure 0-1** in APPENDIX A. This table was used to roughly estimate the biomass concentration from OD results. Then, the volume containing at least 150 mg of biomass was collected from the aquarium with plastic pump to a clear 2L glass beaker. This algal suspension was dewatered to freeze-dried algae powder following the procedures described in Section 4.4. The freeze-dried algae were used for macromolecular content,

CHN and ash moisture content measurements. The details regarding these three tests will be described in Section 4.5.1, 4.5.6, 4.5.7.

#### **4.4 Algal Dewatering**

Algal solution collected directly from the aquariums was dewatered through two steps of centrifugation. In the first step, the fresh algal solution, after collected from aquariums, was centrifuged at 13000 rpm for 10 minutes (RC-5B Refrigerated Superspeed Centrifuge, Sorvall). The supernatant was then removed so that the remaining volume of concentrated algae culture does not exceed 50 ml. Concentrated algal solution was transferred to a 50 ml Falcon tube. The first step is only required when algae culture collected was greater than 1 L. The second step was removing water from algal suspension further and preparing freeze-dried algae, as demonstrated in **Figure 4-5**. First, the concentrated algal suspension was centrifuged at 4000 rpm for 10 minutes (5810R Centrifuge, Eppendorf). After centrifuging, the supernatant was removed with a 3 ml plastic pipette so that the remaining concentrated algae volume in the 50 ml Falcon tube is no more than 5 ml. Then the concentrated algae culture was flash frozen with liquid nitrogen for about 3 minutes and freeze-dried immediately. The 50 ml Falcon tube containing frozen algae was labeled and capped with a piece of Kimwipes fastened by a rubber band. The tube was later placed in a glass container. The container was connected with a freeze-dry machine (Freeze Dry System/Freezone 4.5, Labconco). Freeze-dry process requires 4 days to dehydrate algae completely. Finally, dewatered algal samples

were stored in freezer at -20°C till being used.



**Figure 4-5. Schematic of the freeze-dried sample preparation and analysis processes:** (A) less than 5 ml algae obtained from centrifuging algal suspension; (B) liquid nitrogen which was used to flash freeze centrifuged algae pellets; (C) freeze-dry instrument (Freeze Dry System/Freezone 4.5, Labconco) used to freeze-dry frozen algae; (D) freeze-dried algae in 50 ml Falcon tube; (E) freeze-dried algae stored in the freezer at -20 °C; (F) all tests required to be performed with freeze-dried algae

## 4.5 Sample Analysis

### 4.5.1 Macromolecule

#### 4.5.1.1 Protein

There are two methods to measure Algal protein content. One is estimating by multiplying the determined nitrogen content by a nitrogen-to-protein conversion factor. A

universal conversion factor of 6.25, developed by (Jones, 1931), was used in this study to calculate the protein content. The nitrogen content of the dry biomass was obtained in the CHN test. The CHN test will be described in Section 4.5.6.

The second method determined the protein content by a chemical analysis using lysis buffer (González López et al., 2010). The standard operation method is stated in APPENDIX B. 1 L lysis buffer was made before 20 mg of freeze-dried algal powder was measured and milled using mortar and pestle for 5 minutes. Then ground algae were dissolved into 10 ml lysis buffer to facilitate breaking down the cells to release cellular macromolecular compounds. This mixture was transferred to a 15 ml Falcon tube and placed on a shaker table. The shaker table was operated for 1 hr to facilitate mixing and extraction. After that, 100  $\mu$ l suspension was transferred to a 2 ml centrifuge tube. Different standards made by lysis buffer and BSA protein standards, with concentration ranging from 0 to 0.8 g/L, were also prepared in five of 2 ml centrifuge tubes respectively. The volume of each sample and standard was 100  $\mu$ l. Then each centrifuge tube was added with 0.1 ml SDS solution and 1 ml Reagent C solution and incubated for 10 minutes. After that, additional 0.1 ml Folin Reagent was added to the centrifuge tube and incubated for 30 minutes in dim light. All tubes were centrifuged at 3000 rpm for 1 minute and the supernatant was transferred to a 4.5 ml plastic cuvette. The absorbance of the samples and standards was measured at a wavelength of 750 nm with a spectrophotometer (G10S UV-Vis, Thermo Fisher Scientific). The spectrophotometer

should be zeroed using deionized water before measurement. Samples were processed in the dark to prevent degradation of the Folin reagent. The spectrophotometric absorbance was converted to protein concentration using a calibration curve calculated by the BSA dissolved in the lysis buffer. The protein content of the moisture and ash free biomass was calculated using the **Eq. 4-1**:

$$ProteinContent \left( \%, \frac{w}{w} \right) = \frac{Measuredcarb. \text{ conc. } \left( \frac{mg}{mL} \right) \times 0.1 \text{ ml} \times 100}{20 \text{ mg} \div AshandMoistureContent} \times 100$$

**(Eq. 4-1)**

#### **4.5.1.2 Carbohydrates**

Carbohydrate analysis is based on a pretreatment process and the Anthrone Method which is considered as a precise assay to determine low concentration of carbohydrate in the biomass (Morris, 1948; Morse, 1947). The standard operation method is stated in APPENDIX C. The carbohydrate analysis and the protein measurement have the same pretreatment process for freeze-dried algae, including weighing, milling, dissolving and shaking. 10 ml mixture of milled algae and lysis buffer was sufficient for duplicate measurements in the protein and carbohydrate tests. 0.1 ml of the mixture obtained from the pretreatment process was transferred to a 15 ml Falcon tube, followed by additional 0.9 ml of lysis buffer to make a 10x dilution. The standards, with the concentration between 0 to 0.08 g/L of glucose, were prepared using 2g/L glucose water solution and

lysis buffer. 1 ml of each standard and pretreated sample was transferred to a 15 ml Falcon tube and placed on ice for chilling. Then 2 ml of chilled 75% sulfuric acid and 4 ml of chilled anthrone solution were added to the 15 ml Falcon tube in order. Vortexing was conducted after each addition. After that, all tubes were capped and boiled in 100 °C water for 15 minutes and moved out to cool to the room temperature. A small portion of this solution was transferred to a disposable 4.5 ml plastic cuvette. The absorbance of each was read at 578 nm with the spectrophotometer (G10S UV-Vis, Thermo Fisher Scientific). Carbohydrate concentration was calculated using the calibration curve achieved by standards and the carbohydrate content can be obtained as **Eq. 4-2**:

$$\text{Carbohydrate Content} \left( \%, \frac{w}{w} \right) = \frac{\text{Measured carbo. conc.} \left( \frac{mg}{mL} \right) \times 1 \text{ ml} \times 1000}{20 \text{ mg} \div \text{Ash and Moisture Content}} \times 100$$

(Eq. 4-2)

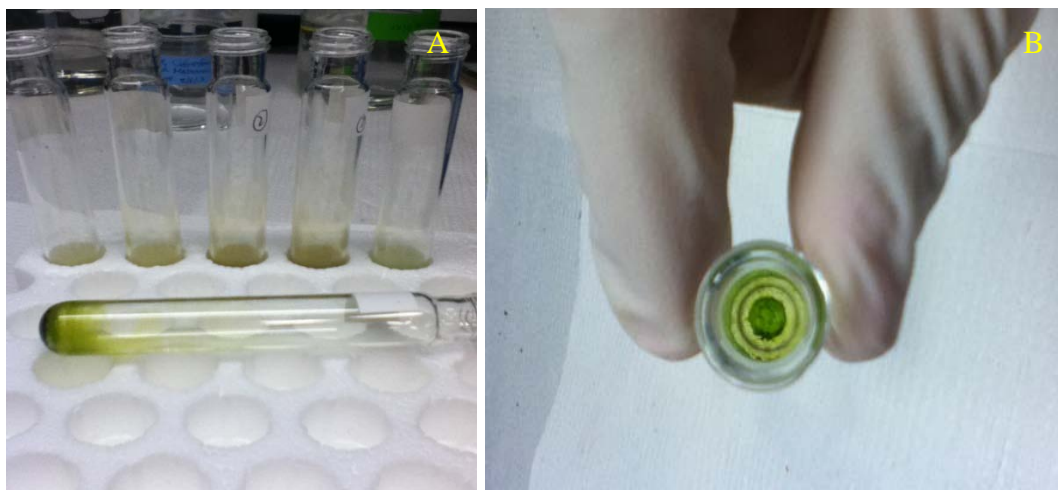
#### 4.5.1.3 Lipids

Algal lipid extraction was performed following a method adapted from the Bligh and Dyer method (Bligh et al., 1959). The lipid extraction was obtained by using chloroform, methanol and water as solvents for different ingredients. 20 mg freeze-dried algae powder was used for duplicate measurements.





**Figure 4-6. Nitrogen evaporator used to evaporate and remove chloroform from chloroform-lipid mixture**



**Figure 4-7. (A) Lipids in a 35 ml glass tube after evaporated; (B) lipids in a 35 ml glass tube after evaporated in a view from the top**

Pretreatment of the lipid test was similar as the pretreatment process in the protein analysis. It included weighing algae, milling algae for 5 minutes, dissolving in 10 ml DI water and transferring the mixture to a 15 ml Falcon tube. 4 ml of the well-mixed

suspension was transferred from the Falcon tube to a 35 ml glass bottle. 5 ml of chloroform and 10 ml of methanol were then added to the glass bottle for extraction. This extraction was operated for 24 hours on a shaker table at the speed of 320 rpm. After that, 5 ml of DI water and 5 ml of methanol were added to the glass bottle. The glass bottle was then centrifuged at 4000 rpm for 10 minutes. Under this solvent ratio, a two-phase (liquid-liquid) system was formed, with water and methanol on the top and chloroform at the bottom. The chloroform layer, containing the extracted lipids, was transferred to a 15 ml pre-weighed glass tube with a Pasteur pipette. After the first time of lipid extraction, another 10 ml of chloroform was added to the same glass bottle to generate the methanol/water-chloroform system for the second time. The bottle was again centrifuged at 4000 rpm for 10 minutes and the lower layer was transferred to the same 15 ml glass tube where the first batch of lipids and chloroform were collected. A nitrogen evaporator (N-EVAP 112, Organomation Associates, Inc., USA) shown in **Figure 4-6** was used to evaporate the chloroform in the glass tube completely. The evaporator also includes a 37 °C water bath at the bottom to facilitate evaporation. After evaporation, the tube was taken out of the water bath and dried. The weight difference of glass tubes before and after lipid extraction was the weight of lipids, as indicated in **Eq. 4-3**. The lipids, shown in **Figure 4-7**, can be further processed into biofuel for transportation or other purpose.

$$\text{Lipid Content} = \frac{\text{Weight difference of glass tube before and after extraction (g)}}{20 \text{ mg biomass} \times \frac{4 \text{ ml}}{10 \text{ ml}}} \times 100\%$$

(Eq. 4-3)

#### 4.5.2 Total Suspended Solids (TSS) and Volatile VSS Solid (VSS)

Total Suspended Solids (TSS) and Volatile VSS Solid (VSS) tests were conducted to determine non-soluble total solids and volatile solids concentration of algal solution. These two tests include 4 steps: filter preparation, sample filtration, sample drying and sample ignition.

Filtration apparatus was set up before starting the tests. 50 ml DI water was measured using a graduate cylinder and filtered with a Whatman GF/F glass fiber filter. After filtration, the filter was ignited at 550 °C in a muffle furnace for 15 minutes to dry completely. The ignited filter was moved out of furnace carefully and then weighed after it cooled down to room temperature. After the blank filter preparation, 200 - 300 ml of fresh algal solution was collected manually with a plastic pump from aquarium for duplicate measurements of TSS, VSS, total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), total alkalinity and dissolved organic carbon (DOC). A stir rod was used to mix the collected algal solution completely. 50 ml of the well-mixed algal solution was measured with a graduate cylinder and filtered with the pre-weighed filter. The same procedure was conducted for another 50 ml of the solution for duplicates. Then,

another 100 ml of the solution was filtered to create sufficient filtrate for the total nitrogen and phosphorus, total alkalinity and DOC tests, which will be discussed in Section 4.5.3, 4.5.5 and 4.5.9, respectively. The glass filter with residue was dried at the temperature between 103 and 105 °C for 24 hours. After drying, the filter was taken out of the oven, cooled and weighed again. The difference between the second time weight measurement (blank) and the first time weight measurement (with dried residue) represents the total suspended solids in the 50 ml algal suspension. Later, dried filter and biomass residue were ignited at 550 °C for 15 minutes in a muffle furnace. The filter was weighed again after ignition and the weight loss during the ignition process equals the amount of volatile solids in the 50 ml water sample. Final TSS and VSS values were calculated by the average of duplicates.

### **4.5.3 Nutrients**

#### **4.5.3.1 Sample Preparation**

The filtrate obtained from the filtration of 200 ml well-mixed algal solution (described in section 4.5.2) was used in TDN, TDP, total alkalinity and DOC tests. 50 ml of filtrate were measured and transferred to a 50 ml Falcon tube for total alkalinity test. Another 50 ml filtrate was measured and transferred as well to provide a duplicate set. The rest of the filtrate was filtrated again using a 20 ml syringe connected to a 0.22 µm Nylon filter. The new filtrate was used to fill two 25 ml EPA DOC glass vials till there

is no air space in the vials. These two vials were used as duplicates in DOC test. Two 50 ml Falcon tubes were used to hold the remaining filtrate for TDN and TDP tests. These sealed water samples could be stored in the refrigerator for about one month before being analyzed.

#### **4.5.3.2 Total Dissolved Nitrogen (TDN)**

TDN and TDP in the medium were measured to indicate physiological condition of microalgae and their nutrient removal efficiency. The method adopted to determine TDN involved a digestion process of oxidizing inorganic and organic nitrogenous compounds to nitrate (*Standard Method 4500-N C. Persulfate Method*) following the colorimetric measurement (*Standard Method 4500-NO<sub>3</sub><sup>-</sup> B. Ultraviolet Spectrophotometric Screening Method*) to quantify nitrate.

3.609 g of dried potassium nitrate (KNO<sub>3</sub>) was dissolved in 1 liter deionized water to make primary standard stock with the concentration of 500 mg-N/L. This primary stock was diluted to 25 mg-N/L working stock which can be stored in the refrigerator for a month. Before measuring the TDN in the filtered sample, this working stock was used to dilute to different concentration with DI water to make six 100-ml standards. Since the concentration of the six TDN standards varied from 0 to 24 mg-N/L, the filtered sample (see Section 4.5.3.1) stored in the 50 ml Falcon tube was diluted to a concentration that is in the range of the concentration of standards. The TDN concentration in the filtered

sample can be estimated by the initial concentration of nitrogen in the medium. 40 ml of each standard and diluted sample was measured and transferred to a 60-ml Pyrex tube. Then, 10 ml of 20 g/L potassium persulfate ( $K_2S_2O_8$ ) and 1 ml of 6 mol/L sodium hydroxide (NaOH) were added to each Pyrex tube and the final water level was marked. As an important part of the digestion method, all samples and standards were autoclaved at 121 °C and 15 psi for 45 minutes to convert all forms of nitrogenous compounds to nitrate in the liquid phase. After samples and tubes were moved out and cooled completely, DI water was added to the 60-ml Pyrex tube till the water level reached the marked line. 1 ml of 7 mol/L HCl was added to the tube later. The absorbance was read at 220 nm and 275 nm with a spectrophotometer (G10S UV-Vis, Thermo Fisher Scientific). Quartz 1-cm path cuvettes were used in this measurement instead of plastic ones for higher accuracy in this short wavelength range. A linear regression curve was established using the nitrogen concentration and absorbance of standards. The curve was used to calculate TDN of all samples.

#### **4.5.3.3 Total Dissolved Phosphorus (TDP)**

Similar to TDN test, all particulate and dissolved organic and inorganic phosphates also need to be acidic persulfate oxidized to inorganic ortho-phosphate before TDP measurement started. Phosphorus standard stock was made by dissolving 0.439 g of dried potassium monobasic phosphate ( $KH_2PO_4$ ) in 1 L DI water. Five 100-ml final standards, with concentration ranging from 0 – 0.7 mg-P/L, were prepared with this stock

solution and DI water. The phosphorus concentration of the samples was estimated based on the initial P concentration in the medium and then diluted to the concentration that is in the range of the concentration of standards. As described in TDN method, 40 ml of each standard and diluted sample were transferred in a 60-ml Pyrex tube. 1 ml of 40 g/L potassium persulfate ( $K_2S_2O_8$ ) was also added to the tube, with the water level marked. After autoclaving at 121 °C and 15 psi for 45 minutes, the tubes were moved out and cooled to room temperature. Lost water during digestion process was replaced by adding DI water to the marked water level. Then 5 ml P-premix reagent, consisting of potassium antimonyl tartrate, ammonium molybdate, concentrated sulfuric acid, ascorbic acid and DI water, was added to the Pyrex tube as well. This mixture was later transferred to a quartz 1-cm path cuvette and its absorbance at 885 nm wavelength was determined by a UV spectrophotometer (G10S UV-Vis, Thermo Fisher Scientific). The concentration of the samples was calculated based on the calibration curve established by the standards. More details can be found in *Standard Methods for the Examination of Water and Wastewater*.

#### **4.5.4 Optical Density**

The optical density (OD) was monitored every day because it has a good linear relationship with biomass concentration. 2 ml of fresh algae suspension was collected from the aquarium and transferred to a 4.5 ml disposable plastic cuvette for absorbance measurement under 600 nm wavelength with a UV-Visible spectrophotometer (G10S

UV-Vis, Thermo Fisher Scientific). DI water was used to zero spectrophotometer before algal sample testing.

#### 4.5.5 Specific Growth Rate

For the batch culture, algae will increase their number at a constant rate in the exponential phase, the rate of growth can be define:

$$r = \left( \frac{dX}{dt} \right) = \mu X \quad (\text{Eq. 4-4})$$

$$\mu = \frac{1}{X} \left( \frac{dX}{dt} \right) \quad (\text{Eq. 4-5})$$

When Equation 4-5 is integrated, the following form results.

$$\mu = \ln \left( \frac{X_2}{X_1} \right) / (t_2 - t_1) \quad (\text{Eq. 4-6})$$

Where  $r$  = rate of microalgae growth based on optical density,  $d^{-1}$

$X$  = concentration of viable microalgae in optical density at time  $t$ ,

$\mu$  = specific growth rate,  $d^{-1}$

$X_1$  and  $X_2$  = optical density of algal culture at time 1 ( $t_1$ ) and time 2 ( $t_2$ ) respectively.

#### 4.5.6 Total Alkalinity (TA)

Alkalinity is an indicator of the capacity of a solution to neutralize acid without causing dramatic change in pH. The total alkalinity, given in the unit of  $\text{mg-CaCO}_3/\text{L}$ , is



defined as the additional quantity of the acid needed to drive the pH of the solution down to 4.2. In this experiment, 50 ml of the filtrate obtained from TSS and VSS tests were measured and transferred to a 50 ml Falcon tube as the total alkalinity sample. Duplicates were required for total alkalinity measurement. This measurement was conducted using a digital titrator (DL 15 Titrator, Mettler Toledo) than traditional titration method for more accurate and consistent results.

#### **4.5.7 CHN**

About 20 mg of the freeze-dried algal samples were dried overnight at 103 – 108 °C in oven to prepare for the CHN test. This test was conducted using an Elemental Analyzer (Series II CHNS/O Analyser 2400, Perkin Elmer). The instrument was warm up for 3 hours. Before real samples testing, several blanks and K samples (composition known) were analyzed to make sure the analyzer functioned well and stably. 2.1 – 4.5 mg of freeze-dried algae sample was required for each measurement. The sample was wrapped in a small piece of aluminum foil with a tweezers and placed a labeled position of the sampler. After the weight and position numbers were input into the program of the analyzer, elementary composition of each sample was analyzed simultaneously. The results are considered constant and reliable only when the variation of C, H and N content of at least three measurements are no more than 0.4% of weight.

#### 4.5.8 Moisture and Ash

SDT Q600 V8.3 Build 101 Thermogravimetric (TGA) analyzer was used to determine the moisture, volatile, and ash content of algal biomass simultaneously. 5-20 mg of freeze-dried algal sample was required for each measurement. The sample was heated at a rate of 10 °C /min under 100 mL/min of N<sub>2</sub> flow until 850 °C. Then heated algae were cooled to 100 °C with dry air flow at 100 mL/min and heated again at the rate of 10 °C /min until 850 °C. The program was set to not move past 110 °C (moisture determination) until mass does not change ( $\pm 0.01$  wt%/min) to determine the moisture content (**Eq.4-4**). The constant weight ( $\pm 0.01$  wt%/min) at 850 °C regions was recorded for calculating the ash content (**Eq.4-5**).

$$\text{Moisture content} = \frac{\text{Initial weight (g)} - \text{weight at 110 °C (g)}}{\text{Initial weight (g)}} \times 100\% \text{ (Eq. 4-7)}$$

$$\text{Ash content} = \frac{\text{Weight at 850 °C (g)}}{\text{Initial weight (g)}} \times 100\% \text{ (Eq. 4-8)}$$

#### 4.5.9 Carbon Species

Considering some molecules can be in the process of being converted from reactants to products or in opposite, ion strength and activity coefficient were introduced in the calculation to adjust the concentration of carbonate species, as shown in **Eq. 4-6** and **Eq.4-7**. The initial concentrations of cations and anions in the medium were used to determine the ion strength that was assumed constant during the algal growth period.

$$I = \frac{1}{2} \sum c_i z_i^2 \text{ (Eq. 4-9)}$$

$$\log \gamma_i = -0.5 z_i^2 \left( \frac{\sqrt{I}}{1 + \sqrt{I}} \right) \text{ (Eq. 4-10)}$$

In these two equations,  $I$  is the ion strength,  $\gamma_i$  is the activity coefficient of ion.  $c_i$  and  $z_i$  are the concentration and charge of  $i$  ion, respectively.

Inorganic carbon consumed by the microalgae in the photosynthesis process was offset by the surface diffusion from the atmosphere and  $\text{CO}_2$  gas cylinder. These carbon species dissolved in the batch culture may include  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ .  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  have an equilibrium relationship in the water. Furthermore, the distribution of these three carbonate species can be described as a function of the pH. Knowing the total alkalinity, pH and activity coefficient, the carbonate species can be quantified with equations in **Table 4-4**.

**Table 4-4. Equilibrium equations and coefficient regarding total alkalinity**

Reactions	Equations	Known <sup>1</sup>
$\text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$	$\text{pK}_{a1} = \text{pH} - \log \frac{\{\text{HCO}_3^-\}}{\{\text{H}_2\text{CO}_3\}}$	$\text{pK}_{a1} = 6.35$
$\text{HCO}_3^- \leftrightarrow \text{CO}_3^{2-} + \text{H}^+$	$\text{pK}_{a2} = \text{pH} - \log \frac{\{\text{CO}_3^{2-}\}}{\{\text{HCO}_3^-\}}$	$\text{pK}_{a2} = 10.33$
-	$\text{Alk} = \{\text{HCO}_3^-\} + 2\{\text{CO}_3^{2-}\} + \{\text{H}^+\} - \{\text{OH}^-\}$	$\text{pH} = -\log\{\text{H}^+\}$

<sup>1</sup>All carbonate reaction constants are based on room temperature of 20 °C.

#### **4.5.10 Organic Carbon**

The duplicate DOC samples were prepared as described in Section 4.5.3.1. These samples in 25 ml EPA glass vials were stored at -4 °C in the refrigerator till they are tested. Before the test, fresh reverse osmosis water was used for blank. A 21% phosphoric acid solution was also prepared immediately prior to torch analysis and used for the cleaning cycles. The standards with the concentration of 0, 1, 2.5, 5, 10 and 50 ppm total organic carbon were prepared using a 500 ppm stock solution (SpectroPure 500 ppm Carbon Standard Organic, Arlington, TX). Each standard was transferred to a 25 EPA glass vial. The instrument used to measure DOC was a Teledyne Tekmar TOC torch. All blanks, standards and samples were placed on the different positions on the sampler of the TOC torch. After sample ID, standard concentration and position were input into the system of the TOC torch, the DOC concentration of all samples were measured and reported automatically.

#### **4.5.11 pH**

30 ml fresh algal solution was transferred from the aquarium to a 50 ml beaker by a 10 ml sterile pipette for pH test. The pH was measured using a Thermo Scientific pH probe (Orion9107 BN, Thermo Scientific) with an electrode and a pH Meter (Accumet AB15 Basic, Fisher Scientific). The meter was calibrated by standard pH 4, 7 and 11 solution once a month for accuracy.

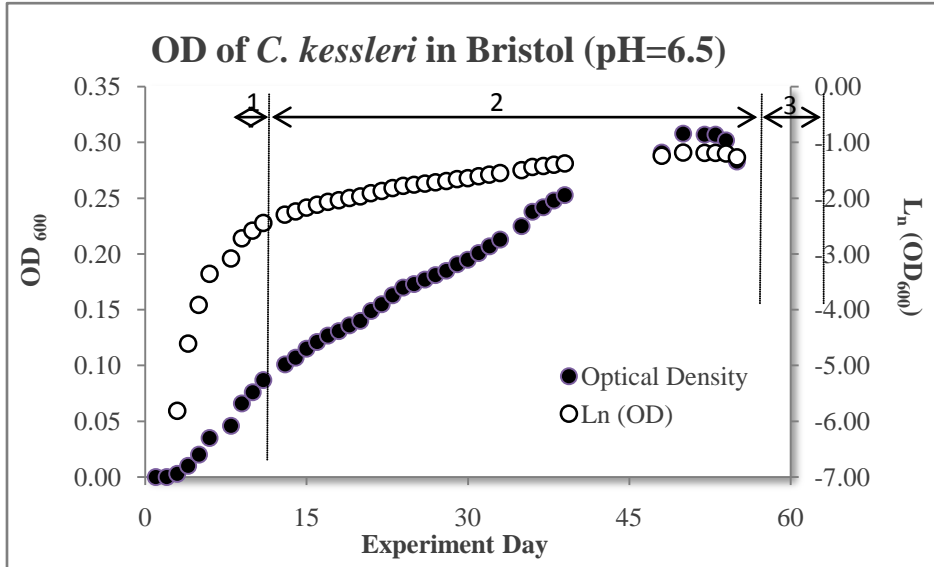
#### 4.5.12 Statistical Analysis

Descriptive statistical analyses of data (i.e., mean, standard deviation) were performed using Microsoft Excel 2007. T-test in statistic analysis was calculated using Microsoft Analysis Tool pack add-in for Microsoft Excel with a critical threshold of 0.05.

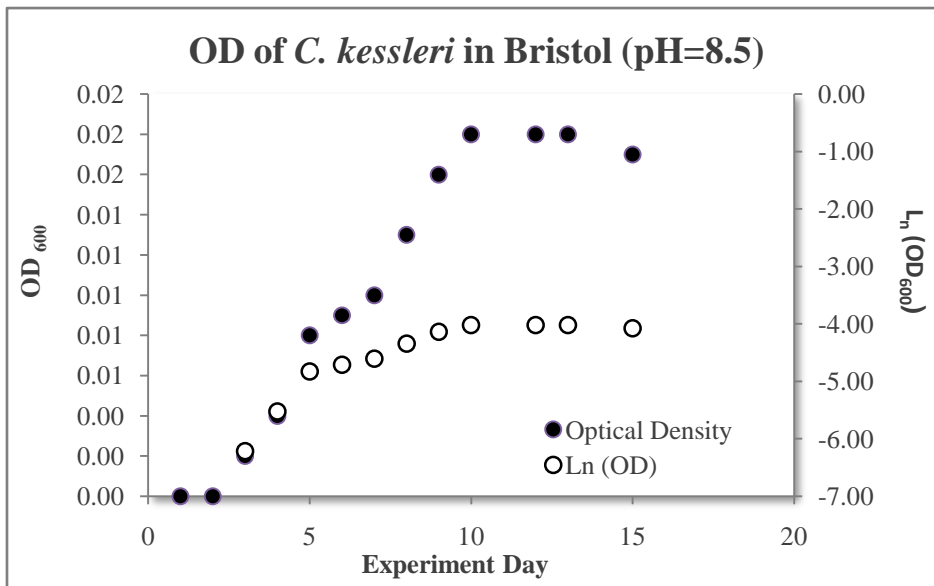
## 5 RESULTS AND DISCUSSION

### 5.1 pH

pH has a strong influence on algal growth and maximum biomass concentration. **Figure 5-1** and **Figure 5-2** demonstrate the change of optical density and specific growth rate of *C. kessleri* in near-neutral (pH 6.5) and alkaline (pH 8.5) Bristol medium along with growth time. Except for pH, other physical parameters were identical for these two batch cultures. Since the optical density is linearly correlated with algal concentration, these figures can be used to represent the relationship of biomass concentration and pH. The highest OD at pH 6.5 reached 0.308, which is 16.6 times greater than at pH 8.5. In addition, neutral pH resulted in higher specific growth rate in the exponential phase and longer growth period. It can be seen that neutral pH is more favored by *C. kessleri* and alkaline pH has a negative effect on algal growth.



**Figure 5-1. OD under 600 nm wavelength and specific growth rate in natural log of OD<sub>600</sub> of *C. kessleri* as a function of time in Bristol medium at pH 6.5. 1, 2 and 3 represent lag phase, exponential phase and stationary phase respectively.**



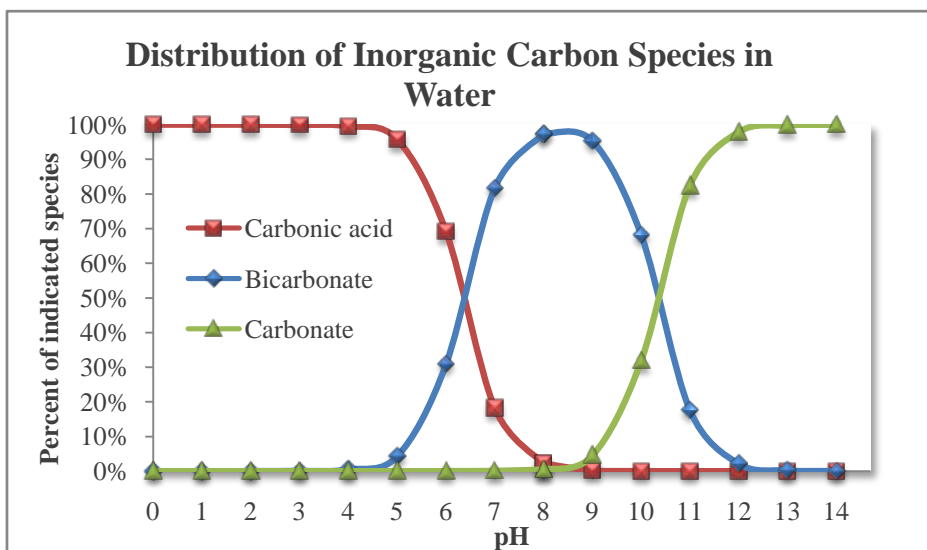
**Figure 5-2. OD under 600 nm wavelength and specific growth rate in natural log of OD<sub>600</sub> of *C. kessleri* as a function of time in Bristol medium at pH 8.5**

The uptake of CO<sub>2</sub> from the medium by the algae drives more HCO<sub>3</sub><sup>-</sup> to form CO<sub>2</sub>, which also releases an OH<sup>-</sup> ion. The accumulation of OH<sup>-</sup> from this process is the main

reason for the increase of surrounding pH that is observed in eutrophic systems. The change of pH may have an impact on the nutrient uptake, substrate and electron transportation, function of membrane, enzyme activity, photosynthesis and respiration processes. High pH can limit the biomass accumulation due to the reduction of biologically available inorganic carbon species including carbon dioxide ( $\text{CO}_2$ ) and bicarbonate ( $\text{HCO}_3^-$ ). Microalgae cannot metabolize  $\text{CO}_2$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  evenly. Carbon dioxide is easy to be taken up by algae as a raw carbon resource in the photosynthesis process. Part of bicarbonate can be metabolized by many microalgal species as well (Talling, 1976). When pH is close to 7, the carbonate species dissolved in the water are mostly carbon dioxide and bicarbonate. High pH can shift the distribution of the carbon species to more  $\text{CO}_3^{2-}$  and  $\text{HCO}_3^-$  and less  $\text{H}_2\text{CO}_3$ , and thus reduce the available biological carbon resource for microalgal growth. **Table 5-1** and **Figure 5-3** demonstrate the relationship between carbonate species distribution and pH in microalgae bioreactors. Related reactions and equations are shown in **Table 4-3**.

**Table 5-1. Distribution of carbonate species in water at pH 6.5 and pH 8.5**

	Carbon Species		
	$\text{H}_2\text{CO}_3$	$\text{HCO}_3^-$	$\text{CO}_3^{2-}$
pH 6.5	43.7%	56.3%	0.0%
pH 8.5	1.1%	96.4%	2.5%

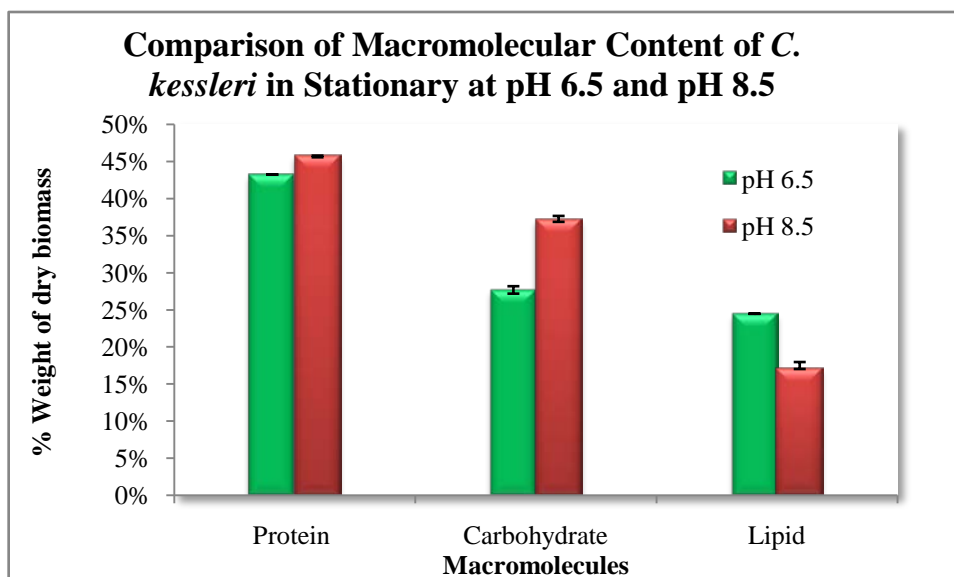


**Figure 5-3.** Distribution of inorganic carbon species as a function of pH at equilibrium in a closed system

For green algae,  $\text{CO}_2$  can be actively transported across not only cytoplasmic membrane but also the single membrane of chloroplast envelope (Goyal et al., 1989). This finding was proved by some other studies later that reported an efficient carbon dioxide and bicarbonate transport system at the chloroplast envelopes of green algae (Badger et al., 1994; Moroney et al., 1991).

The difference of surrounding pH in the two batch cultures resulted in a difference in the algal membrane transport system and pH regulation process, thus leading to a variation in biochemical composition, growth rate and extracellular production. Lipid accumulation was observed in both conditions during the cultivation period. In the stationary phase lipid content achieved 24% at pH 6.5 and 14% at pH 8.5 (see **Figure 5-4 and Table 5-2**). High pH also promoted carbohydrate production from 28% at pH 6.5 to 37% at pH 8.5.





**Figure 5-4.**Macromolecular content comparison of *C. kessleri* in the stationary phase in Bristol medium with pH 6.5 and 8.5. Error bars stands for 2×SD (standard deviation).

**Table 5-2.**Main parameters of growth and biochemical components of *C. kessleri* in Bristol medium at different pH

		pH 6.5		pH 8.5
Growth Period	Day	55		15
Growth Phase	-	Exponential	Stationary	Stationary
OD <sub>600</sub>	-	0.191	0.283	0.017
TSS	mg/L	104.00 ± 0.00	168.00 ± 0.00	13.00 ± 0.00
VSS	mg/L	100.00 ± 2.83	140.00 ± 1.41	13.00 ± 2.83
Protein Content	% dw <sup>1</sup>	57.79 ± 0.00	69.53 ± 0.99	73.93 ± 1.96
Carbohydrate Content	% dw	34.94 ± 6.17	27.68 ± 0.50	37.19 ± 0.46
Lipid Content	% dw	19.33 ± 1.73	24.47 ± 0.00	17.02 ± 0.94

<sup>1</sup>dw represents dry weight biomass

It has been proved that most green algae maintain a constant internal alkaline pH to allow diffusion uptake of dissolved CO<sub>2</sub> (Coleman et al., 1981). The surrounding CO<sub>2</sub> was not sufficient for algae when pH exceeded 8.0. High pH can not only limit the

inorganic carbon resource for algae but also change the function and composition of the cytoplasmic membrane. Taraldsvik(Taraldsvik et al., 2000) reported that high pH affects the metabolic processes by stimulating active inorganic carbon uptake system since algal cells are not capable of maintaining an internal alkaline pH. His study found thatmarine algae species in alkaline medium with pH higher than 8.5 can produce more mono- and poly-saccharides and transport these carbohydrates extracellularly. These carbohydrates are used for participating in metabolic processes, self-preservation and primary production utilized by bacterioplankton(Taraldsvik et al., 2000). In this experiment, higher carbohydrate content was found in *C. kessleri* at pH 8.5than at pH 6.5. Compared with pH 6.5, the majority of algae were found attached onto the inside wall of aquarium with pH 8.5 rather than suspending in the water, due to more extracellular saccharides being released. This might be a survival mechanism for algae to minimize water shear force and exchange resourceswithin the community. Meanwhile, this increased production of carbohydrates may play a crucial role in providing materials and energy in active carbon transport across cytoplasmic membranes in pH 8.5 medium.

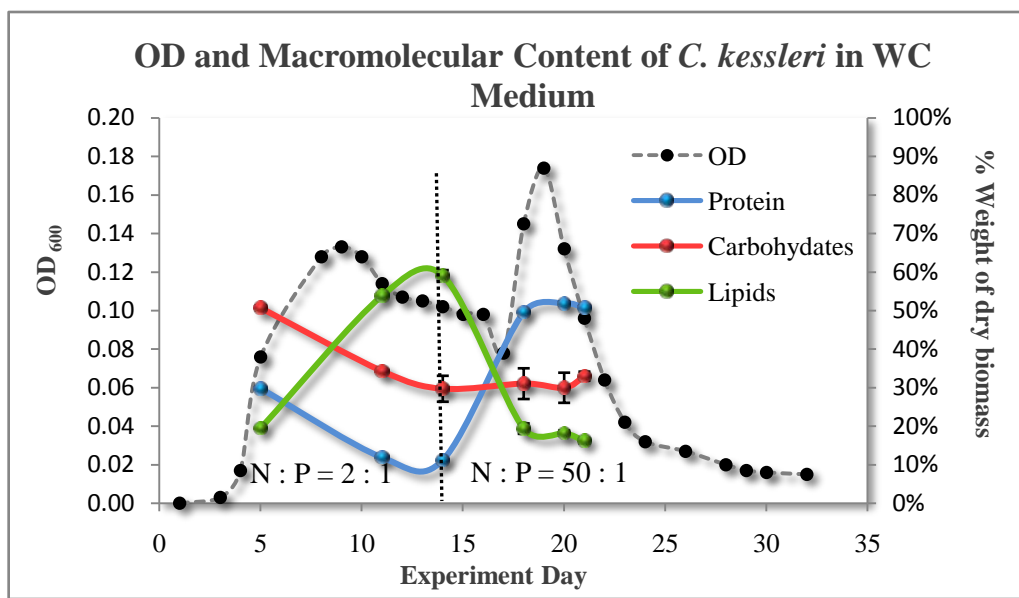
## **5.2 N:P Ratio**

In experiment 2, *C. kessleri* was cultivated in Batch 1 in WC medium with N:P = 2:1. Then  $\text{NaNO}_3$  and  $\text{K}_2\text{HPO}_4$  stocks and water were added to bring the volume up to 144L and shift the N:P ratio from 2:1 to 50:1 by moles. The new batch with N:P = 50:1 had the same initial phosphorus concentration with the last one. This batch was the Batch 1 in

experiment 3. The *C. kessleri* continuously grew in the same aquarium from N:P = 2:1 to N:P = 50:1.

The growth of *C. kessleri* species in conditions was investigated to study its biochemical response to different N:P ratios. Since the Redfield ratio of biomass is C:N:P = 106:16:1, the N:P ratios of 2:1 and 50:1 are used in this experiment to represent insufficient and sufficient nitrogen condition, respectively. It was observed that the lower N:P ratio (2:1) limited algal growth and caused a 20% reduction of the peak biomass concentration compared to the higher N:P ratio (50:1), as illustrated in **Figure 5-5**. In addition, the OD curve indicates a sharper variation of biomass concentration in the stationary phase in N-sufficient condition (N:P = 50:1) than that in N-limited condition (N:P = 2:1). All these findings demonstrate that sufficient supply of nitrogen can help *C. kessleri* thrive in the WC medium and accelerate the algal metabolism rate.

N-limited condition stresses algal growth, causing lower biomass concentration in the stationary phase compared with N-sufficient condition, as seen in **Figure 5-5**. The figure also shows that this lower biomass concentration also accompanies low protein content under the N-limited condition. This is because insufficient inorganic nitrogen in the medium will decrease the supply of amino acids and slow down the translation of mRNA, thereby reducing the protein synthesis and cell division rate.



**Figure 5-5.** OD<sub>600</sub> and macromolecular content of *C. kessleri* as a function of time in WC medium with different nitrogen to phosphorus ratio. Error bars stands for 2×SD (standard deviation).

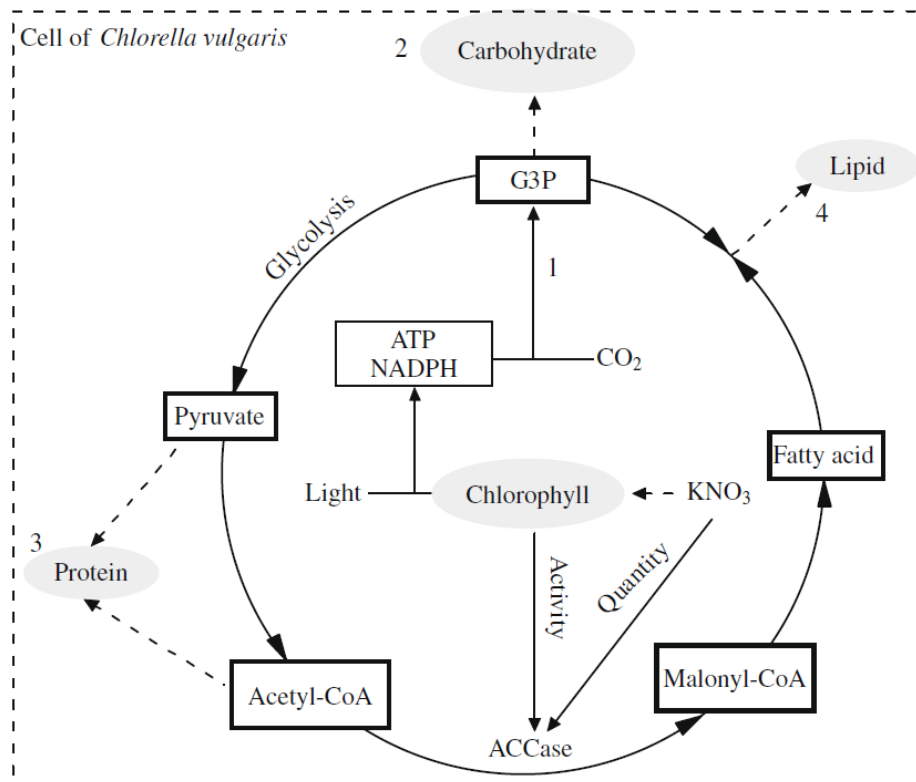
**Figure 5-5** indicates the variation of biochemical compositions of *C. kessleri* under N-sufficient and N-limited conditions. It is obvious that not only nitrogen to phosphorus ratio in the medium but also the algal physiological state can significantly affect algal biochemical components including protein, lipids and carbohydrates. Protein content increased 2.4 times from N:P = 2:1 to N:P = 50:1 in the stationary phase. The difference of protein content was caused by the difference of external inorganic nitrogen concentration. Deficiency of nitrogen inhibited the synthesis of proteins.

There is a significant decrease of protein content from lag phase to stationary phase under N-limited condition, as can be seen in **Figure 5-5**. In the exponential phase large amount of protein is produced in algal cells to promote cell growth and assist DNA

replication. However, in the stationary phase the metabolic rate becomes relatively slow, due to the appearance of severe deficiency of nitrogen in N-limited medium.

Without sufficient nitrogen, lipid content had a significant increase from 20% in the exponential phase to 54% in the stationary phase ( $p < 0.05$ ). A 36% reduction of lipid content was observed in N:P = 50:1 compared with N:P = 2:1. Insufficient nitrogen and low growth rate can be the key factors that result in an increase in lipid content. Lipid content for N-sufficient *C. kessleri* maintained a low level of 18% without a significant change from exponential phase to stationary phase ( $p > 0.05$ ).

Cell growth and biochemical compound synthesis are closely related with each other. Besides, the concentration of biochemical compounds is significantly influenced by the quantity and activity of related enzymes. This complex biosynthetic process in *C. kessleri* species is illustrated in **Figure 5-6**. According to this routine, the crucial step in synthesizing lipids (fatty acids) is considered as the reaction in which acetyl-CoA is converted to malonyl-CoA catalyzed by ACCase (Lv et al., 2010). Previous research (Sukenik et al., 1991) has proved that in nitrogen limited environment, a reduction in the quantity of cellular ACCase occurs due to the lack of nitrogen as raw material for producing protein. However, lipid accumulation in algal cells is still in process even though the cell division ceased. With no need to separate lipids to daughter cells, low cell division rate allows lipids to accumulate in cells.



**Figure 5-6. Essential macromolecules biosynthetic processes in cells of *C. vulgaris* (Lv et al., 2010)**

Although deficiency of nitrogen in the medium can lead to lipid accumulation in individual algal cell, algal growth is slowed down simultaneously. High growth rate and high lipid content can hardly be fulfilled at the same time to achieve the theoretical maximum lipid productivity. However, other optimal conditions can be added into the cultivation process to promote the activity of ACCase and protein synthesis, such as feeding with carbon dioxide and increasing light intensity.

The change of the uptake of nitrogen and phosphorus for algae can be represented using the removal of nitrogen and phosphorus from the medium. The two batches with WC medium of different N:P ratios described in **Table 5-2** were set up with the same

phosphorus concentration. However, the removal of phosphorus in the stationary phase of these two batches was significantly different, as summarized in **Table 5-3**. Even though the batch with N:P = 2:1 showed a greater nitrogen removal efficiency compared with N:P = 50:1, *C. kessleri* in the N:P = 50:1 performed much better in absolute removal of nitrogen from the medium. The ratio of absolute nitrogen and phosphorus removal for the batch with N:P = 50:1 was higher than the Redfield Ratio (N:P = 16:1), indicating an excessive uptake of nitrogen in N-sufficient culture. This excessive consumption of nitrogen can accelerate protein synthesis to increase the quantity and activity of the enzymes for growth and replication. Increased cell density leads to even more nitrogen consumption.

**Table 5-3. Nitrogen and phosphorus removal at N:P = 2:1 and 50:1 (by moles) in WC medium grown with *C. kessleri***

	N:P in the medium	
	Batch 1(WC)	Batch 2(WC)
	N:P = 2:1	N:P = 50:1
Initial N concentration in the medium (mol/L)	0.1	2.5
Initial P concentration in the medium (mol/L)	0.05	0.05
N removal	90%	27%
P removal	71%	39%
Absolute N removal <sup>1</sup> (mol/L)	0.09	0.67
Absolute P removal <sup>2</sup> (mol/L)	0.04	0.02
The ratio of absolute N and P removal	2.33	38.52

<sup>1</sup>Absolute N removal = Initial N concentration × N removal

<sup>2</sup>Absolute P removal = Initial P concentration × P removal

It is also showed in **Table 5-3** that low N:P ratio enhances the phosphorus removal efficiency. Since the initial phosphorus concentration was the same for both treatments,

the absolute phosphorus removal is equal to the phosphorus removal. The ratio of the absolute removal of nitrogen and phosphorus in N-limited medium is significantly less than that in N-sufficient medium, and lower than Redfield Ratio N:P = 16:1 as well. This result suggests an excessive consumption of phosphorus occurred in the N-limited culture. Since the deficiency of nitrogen can limit the biosynthesis process and biomass accumulation, algae need to uptake more phosphorus and other available inorganic elements to synthesize energy storage materials like lipids to survive in the nutrient limited condition.

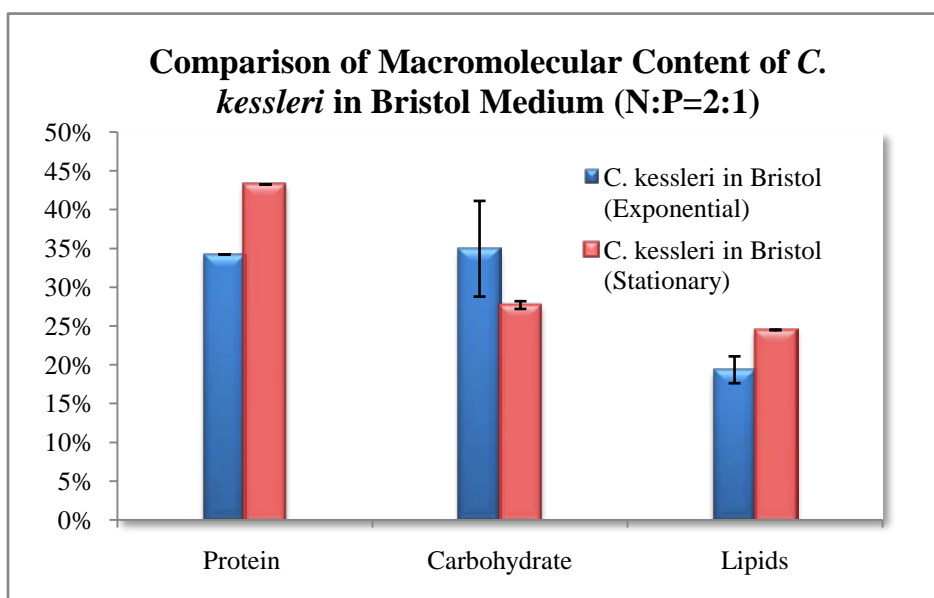
### 5.3 Media Composition

There are many different types of media for freshwater microalgae like SE medium, Bristol medium, BG-11 medium, WC medium, etc. Although their recipes are different, all of them contain basic elements required for the growth of freshwater microalgae. Bristol medium and WC medium are widely utilized for growing freshwater phytoplankton including green algae. In this study, these two types of media were used to cultivate batch cultures of *C. kessleri*, and they were compared with each other based on the ability to promote biomass and lipid yield. The ingredients and concentrations of these two types of media are listed in **Table 4-1** and **Table 4-2** in Section 4.1 Strains and Media. The N:P ratio (2:1) of both types of media was kept constant, as well as other physical cultivation conditions including light cycle, light intensity, water temperature, pH, etc. With the same initial N:P ratio, it is possible to study the effects of other inorganic nutrient in these two types of medium on algal growth and lipid yield.

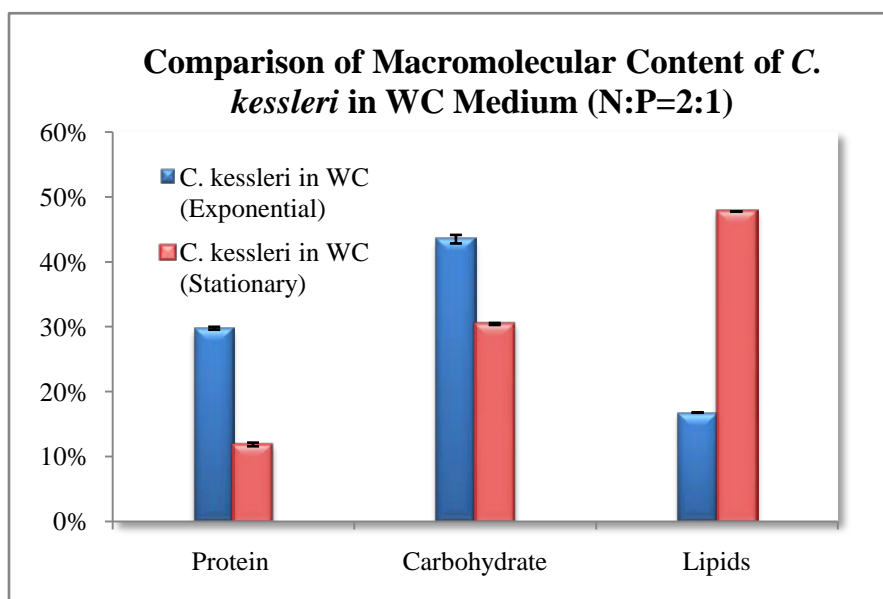


*C. kessleri* reached stationary phase in Bristol medium after 55 days of cultivation, as compared with only 10 days for WC medium. The specific growth rates in exponential state were  $0.81\text{ d}^{-1}$  and  $1.61\text{ d}^{-1}$  in Bristol and WC medium, respectively. This lower specific growth rate is another evidence of the growth retardation in Bristol medium. Although the growth of *C. kessleri* was slowed down in Bristol medium, the maximum VSS obtained in the stationary phase in this batch was  $140\text{ mg/L}$ , 2.3 times greater than that achieved in WC medium. These results indicate that *C. kessleri* in the WC medium has a higher growth activity than in the Bristol medium. This high growth activity accelerates the process of consuming nutrient and contributes to the short growth period in WC medium.

Other than the difference in algal growth, the distribution of cellular biochemical compounds in algal cultivated in Bristol and WC media differed. According to **Figure 5-7**, there was no significant variation ( $P > 0.05$ ) in carbohydrates and lipid content from exponential phase to stationary phase for the culture in Bristol medium, while algae fed with WC medium had a significant variation ( $P < 0.05$ ) in the content of all three biochemical components between these two phases. The dramatic decrease in protein content from exponential phase to stationary phase in WC medium, as shown in **Figure 5-8**, indicates that nitrogen may be the limiting factor for this batch culture. In the WC medium with N:P = 2:1, lipid content increased 1.7 times from exponential phase to stationary phase, caused by the extreme deficiency of nitrogen in the stationary phase.



**Figure 5-7.** Distribution of biochemical component of *C. kessleri* in exponential and stationary phases in Bristol medium with N:P = 2:1 (by moles). Error bars stands for 2×SD (standard deviation).



**Figure 5-8.** Distribution of biochemical component of *C. kessleri* in exponential and stationary phases in WC medium with N:P = 2:1 (by moles). Error bars stands for 2×SD (standard deviation).

In addition, there was almost no change in the concentration of nitrogen and phosphorus in the Bristol medium as can be seen in **Figure 5-7**, because the consumption of nitrogen and phosphorus by algae is negligible compared to the initial high concentration of nitrogen and phosphorus. Meanwhile, the protein content had an increase from exponential phase to stationary phase for the algae cultivated in the Bristol medium. These observations indicate that nitrogen is not the limiting factor in the Bristol medium in stationary phase. Consequently, for algae grown in Bristol medium, the limiting factor that appeared at the end of the log phase may not be the deficiency of nitrogen.

The possible limiting factors for Bristol medium are the micronutrient. Bristol medium included high concentrations of nitrogen and phosphorus; however, it lacked some elements necessary for growth of microalgae, such as Fe, Zn, Si and etc. It was observed that iron makes an important contribution to enhancing the photosynthesis productivity and chlorophyll concentration, as well as the growth rate (Tiamiyu, 2011). Iron ions derived from ferric compounds are indispensable in synthesizing some crucial enzymes and organic products that support healthy algal growth. Previous study also reported retardation of growth and decrease of enzyme activity due to the lack of Zn and Cu (Bartlett et al., 1974). In addition,  $\text{Na}_2\text{SiO}_3$ , and  $\text{Na}_2\text{CO}_3$  and  $\text{Na}_2\text{EDTA}$  added in WC medium, but absent in Bristol medium, were able to buffer the system to maintain a relatively constant pH environment for microalgae. Besides, EDTA can especially

increase the capacity of the medium to carry more microelements like trace metals due to its unique structure.

The second possible factor in limiting the biomass in the Bristol medium is the self-shading effect. As the cell density gradually increased, it is more and more difficult for the light at the back of the aquarium to penetrate through the whole water body. Meanwhile, it was observed that some algae attached to the back wall of the aquarium to obtain light resource as much as possible. As a result, lighting was partially blocked by the algae on the back wall. The severe light deficiency finally led to the reduction of chlorophyll and biomass concentration.

High dissolved organic carbon (DOC) concentration at the end of the log phase is possibly another reason for the growth limitation in the Bristol medium. In the aquarium with Bristol medium, DOC increased along with biomass concentration, reaching the maximum in the stationary phase. Beyond certain level of concentration, DOC may inhibit algal growth due to the shading effect. More details can be found in Section 5.5.

TSS includes both dry biomass and inorganic matters, while VSS only consists of the organic part of the TSS. The difference of TSS and VSS represents the ash content of freeze-dried algae sample. There was a sharp increase in ash content of *C. kessleri* from late exponential phase to stationary phase in Bristol medium as shown in **Table 5-4**. As discussed in Section 5.2, limited nitrogen can lead to excessive

consumption of phosphorus in cells as a surviving mechanism. On the other hand, excessive uptake of nitrogen was also observed in the N-sufficient condition. It was found that rather than nitrogen diffusion or nitrogen active uptake process, incorporating nitrogen into organic matter is the rate-limiting step in nitrogen metabolic process (Conover, 1975). In fact, most of excess nitrogen might stay in forms of inorganic nitrogen or free amino acids. This inorganic portion of nitrogen and phosphorus stored in cells might partially contribute to ash fraction increase.

**Table 5-4. Variation of OD<sub>600</sub> and ash content of *C. kessleri* with time in pH 6.5 Bristol medium with N:P = 2:1 (by moles)**

Expt. Day	<i>C. kessleri</i> in Bristol medium					
	5	10	17	29	38	55
OD <sub>600</sub>	0.020	0.076	0.127	0.191	0.248	0.283
Ash Content (% dw <sup>1</sup> )	4.35%	5.89%	4.73%	4.47%	10.67%	13.97%

<sup>1</sup>dw represents dry weight biomass

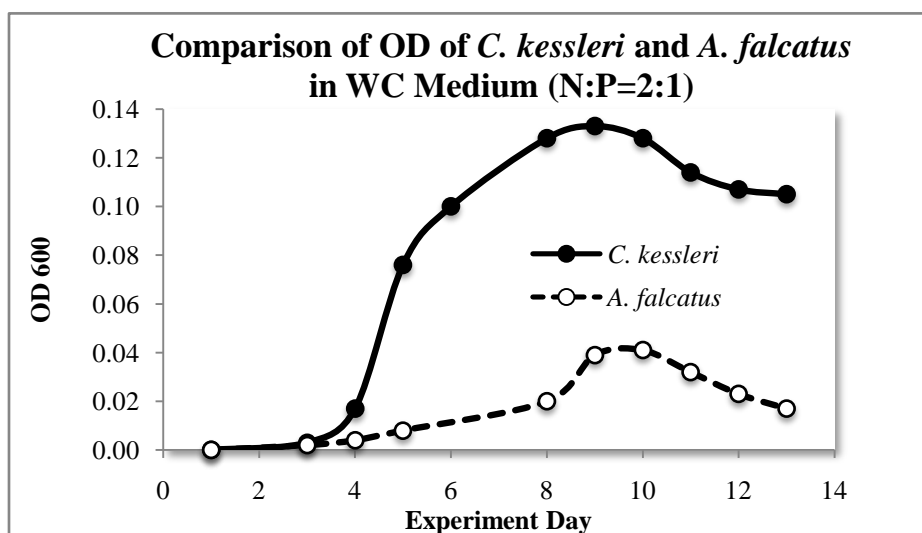
It is inferred that inorganic nutrient other than nitrogen and phosphorus, such as inorganic carbon and micronutrient, started to be stored at the end of the log phase when the limiting factor appeared. The stressful environment might stimulate algae to excessively uptake inorganic nutrient for survival. The excess inorganic nutrient uptake can be another source of the excess ash content.

## 5.4 Algae Strains

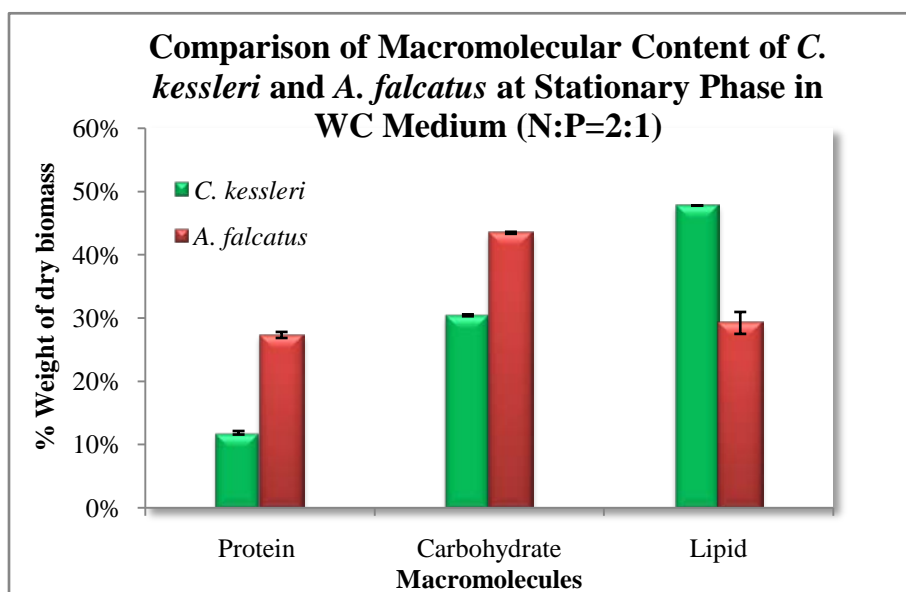
As two genera of single-cell green algae, *C. kessleri* and *A. falcatus* have different

shapes and structures. *C. kessleri* cells are in spherical shape while *A. falcatus* is featured with fiber-shaped cells. Unlike *C. kessleri*, unicellular *A. falcatus* cells can be found in clusters. Both of them are considered as stock cultures with great potential of producing biodiesel (Kalita et al., 2011; Scott et al., 2010).

The growth of *C. kessleri* was compared with that of *A. falcatus* in the pH 6.5 WC medium, with N:P = 2:1 and N:P = 50:1 by moles, respectively. As shown in **Figure 5-9**, in the N-limited treatment, *A. falcatus* experienced poor growth, with its maximum optical density at only 31% of the maximum density of *C. kessleri*. Despite the difference in growth rate, these two species all showed an increase of lipid content in response to nitrogen limitation as can be seen in **Figure 5-10** and **Figure 5-12**. The lipid content in *C. kessleri* achieved a maximum of 54% of dry weight in the stationary phase while the maximum lipid content of *A. falcatus* was 28%. *A. falcatus* obtained more carbohydrates and protein than *C. kessleri*.



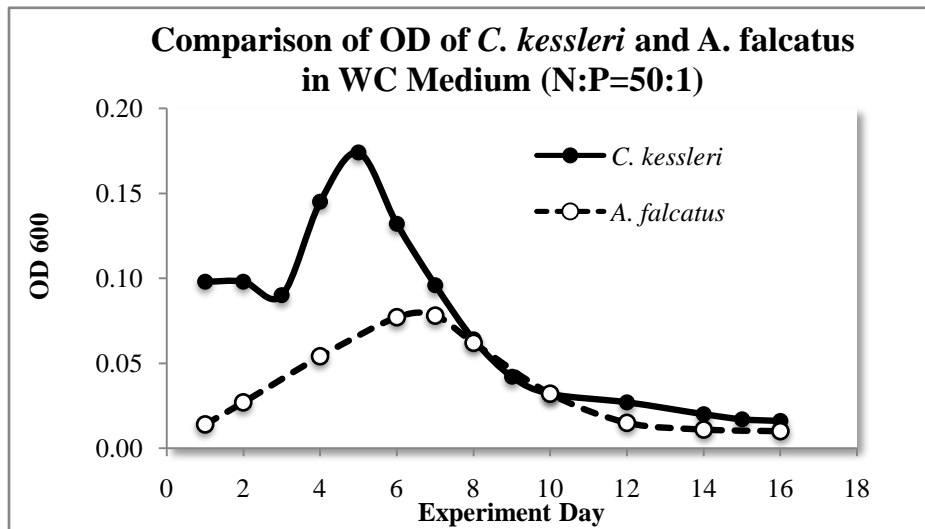
**Figure 5-9.**OD<sub>600</sub> comparison of *C.kessleri* and *A. falcatus* in pH 6.5 WC medium with N:P= 2:1 (by moles)



**Figure 5-10.**Comparison of macromolecular content of *C.kessleri* and *A. falcatus* in pH 6.5 WC medium with N:P=2:1 (by moles). Error bars stands for 2×SD (standard deviation).

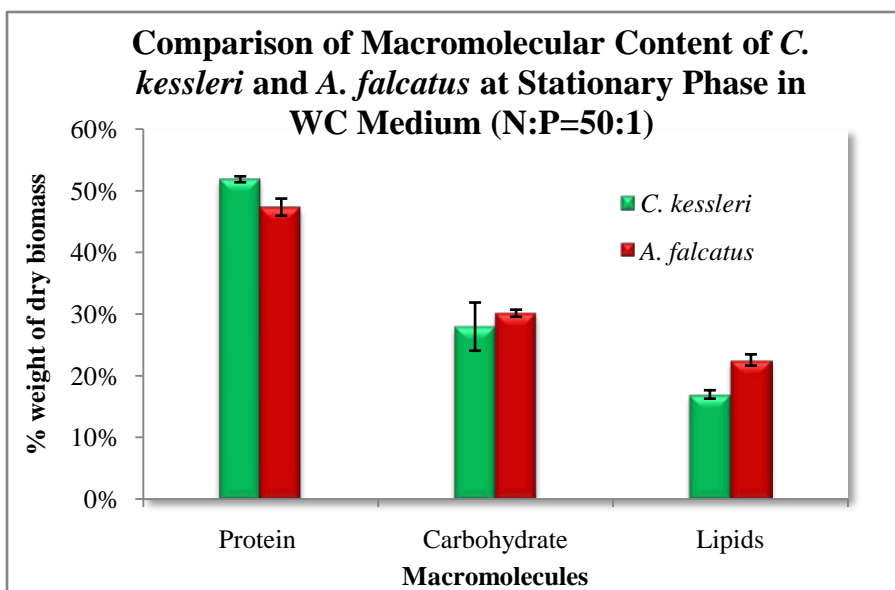
As can be seen in **Figure 5-9**,WC medium with sufficient nitrogen is more favored by *C. kessleri* than *A. falcatus* since *C. kessleri* had higher biomass concentration and

more uptake of nitrogen and phosphorus than *A. falcatus*. As *C. kessleri* quickly thrived in WC medium, nitrogen was drastically reduced, and this resulted in high lipid content in the stationary phase shown in **Figure 5-10**. For *A. falcatus*, less depletion of nitrogen led to lower lipid content and higher protein content in the stationary phase compared with *C. kessleri*. In the steady state, *A. falcatus* is more likely to synthesize and store carbohydrates rather than lipids as an energy storage material in this cultivation condition. In general, *C. kessleri* had 3.7 times higher biomass concentration and 1.9 times higher lipid content compared with *A. falcatus* in the stationary phase. Therefore *C. kessleri* performed much better than *A. falcatus* in terms of lipid productivity in absence of nitrogen.



**Figure 5-11.** OD<sub>600</sub> comparison of *C. kessleri* and *A. falcatus* in pH 6.5 WC medium with N:P=50:1 (by moles)





**Figure 5-12.** Comparison of macromolecular content of *C. kessleri* and *A. falcatus* in pH 6.5 WC medium with N:P = 50:1 (by moles). Error bars stand for 2×SD (standard deviation).

Figure 5-11 and Figure 5-12 clearly show that N-sufficient condition in WC medium has the same effect on both *C. kessleri* and *A. falcatus*, increasing the biomass and protein concentration and decreasing the lipid concentration in the stationary phase compared with N-limited condition. From N:P = 2:1 to N:P = 50:1, the protein content increased 337% while the lipid content dropped 66% for *C. kessleri*, shown in Table 5-5 and Table 5-6. As for *A. falcatus*, the protein content increased 24% while the lipid content dropped 61%. As the environment turned to be in favor of growth when nitrogen became adequate, carbohydrates stored in the *A. falcatus* cells were utilized to produce more protein to facilitate metabolism and biomass accumulation. *C. kessleri* cultivated in the WC medium with N:P = 50:1 had almost the same lipid content as *A. falcatus* but double the biomass concentration in the stationary phase. This indicates that *C.*

*kessleri* has a greater potential in biomass and lipid yield than *A. falcatus* in WC medium with N:P = 50:1.

**Table 5-5. Main parameters of growth and biochemical components of *C. kessleri* and *A. falcatus* in WC medium with N:P = 2:1**

	Unit	<i>C. kessleri</i>		<i>A. falcatus</i>
Growth Period	day	14		22
Growth Phase	-	Exponential	Stationary	Stationary
OD <sub>600</sub>	-	0.076	0.114	0.032
TSS	mg/L	35.00 ± 1.41	66.00 ± 2.83	20.00 ± 2.83
VSS	mg/L	33.00 ± 0.00	60.00 ± 2.83	16.00 ± 2.83
Protein Content	% dw <sup>1</sup>	29.77 ± 0.22	11.86 ± 0.28	27.32 ± 0.48
Carbohydrate Content	% dw	50.70 ± 0.66	34.29 ± 0.15	43.46 ± 0.16
Lipid Content	% dw	19.53 ± 0.00	53.85 ± 0.00	29.22 ± 1.73

<sup>1</sup>dw represents dry weight biomass

**Table 5-6. Main parameters of growth and biochemical components of *C. kessleri* and *A. falcatus* in WC medium with N:P = 50:1**

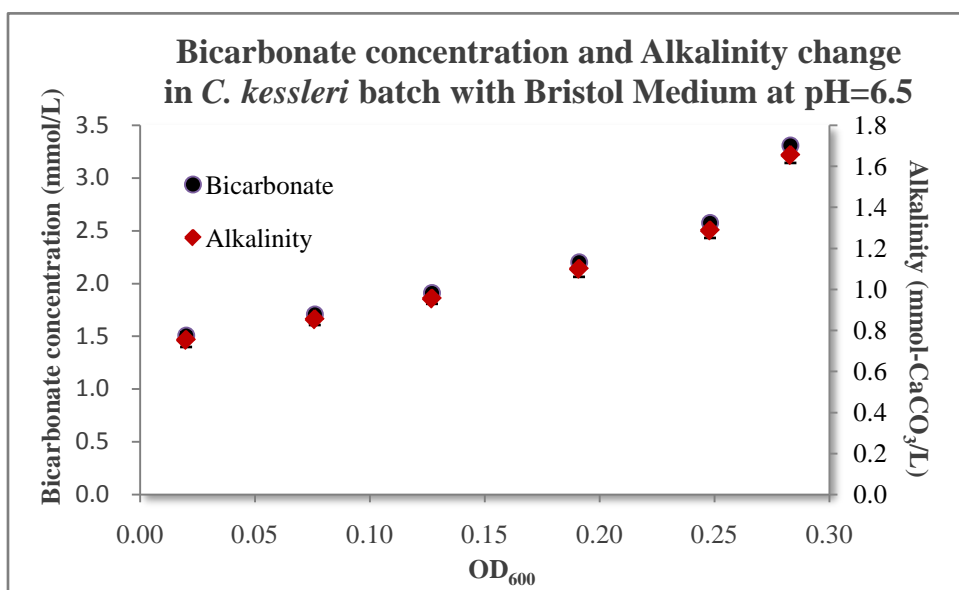
	Unit	<i>C. kessleri</i>		<i>A. falcatus</i>
Growth Period	day	18		16
Growth Phase	-	Exponential	Stationary	Stationary
OD <sub>600</sub>	-	0.145	0.132	0.077
TSS	mg/L	75.00 ± 1.41	62.00 ± 2.83	37.00 ± 1.41
VSS	mg/L	65.00 ± 1.41	58.00 ± 2.83	33.00 ± 1.41
Protein Content	% dw <sup>1</sup>	49.59 ± 0.22	51.84 ± 0.49	47.33 ± 1.39
Carbohydrate Content	% dw	31.04 ± 3.99	29.97 ± 3.91	30.13 ± 0.58
Lipid Content	% dw	19.37 ± 1.35	18.18 ± 0.69	22.54 ± 0.92

<sup>1</sup>dw represents dry weight biomass

## 5.5 Alkalinity and DOC

The variation of total alkalinity in the Bristol medium was observed in this study to

investigate the buffer capacity change of the medium during cultivation period and its effect on algal growth. For the batch culture of *C. kessleri* at pH 6.5, the alkalinity in the aquarium increased from 75 to 165 mg-CaCO<sub>3</sub>/L during the growth period, as shown **Figure 5-13**. At the same time, the concentration of bicarbonate increased 119% from lag phase to steady phase in the Bristol medium. **Table 5-7** indicates bicarbonate was the main resource that contributes to alkalinity. The equations regarding the calculation of carbon species distribution and total alkalinity can be found in **Table 5-1** and **Eq. 2-2**. Ion strength, only introducing 0.03% error at maximum, was ignored in the calculation of carbon species concentration.



**Figure 5-13.** Variation of bicarbonate concentration and total alkalinity with OD<sub>600</sub> in Bristol medium grown with *C. kessleri* at pH = 6.5. Error bars stand for 2×SD (standard deviation).

**Table 5-7. Change of total alkalinity and bicarbonate concentration as a function of time in Bristol medium at pH 6.5**

Experiment				
Day	ALK <sub>TOT</sub> <sup>1</sup>	HCO <sub>3</sub> <sup>-</sup>	$\Delta(\text{ALK}_{\text{TOT}} - \text{HCO}_3^-)$	$\Delta(\text{ALK}_{\text{TOT}} - \text{HCO}_3^-)/\text{ALK}_{\text{TOT}}$
-	(mEq/L)	(mEq/L)	(mEq/L)	(mEq/L)
5	1.51	1.51	1.05E-03	0.07%
10	1.71	1.71	1.48E-03	0.09%
17	1.91	1.91	1.61E-03	0.08%
29	2.20	2.20	6.24E-04	0.03%
38	2.57	2.57	9.13E-04	0.04%
55	3.31	3.31	1.16E-03	0.04%

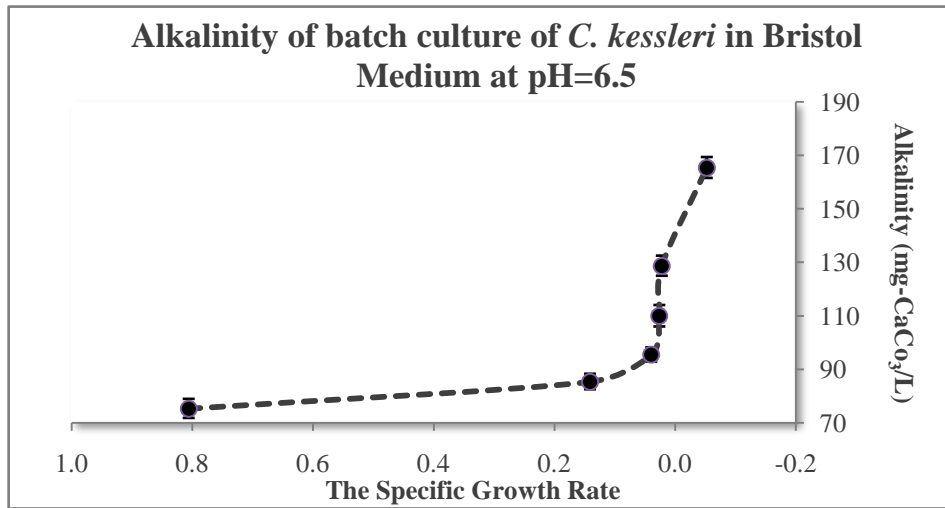
<sup>1</sup>ALK<sub>TOT</sub> stands for total alkalinity

Since pH of the medium was monitored by the smart pH meter and maintained at pH 6.5, the concentration distribution of carbonic acid, bicarbonate and carbonate did not change throughout the experiment. As *C. kessleri* biomass started to thrive in the Bristol medium, the accelerated uptake of CO<sub>2</sub> forced more CO<sub>2</sub> being supplied to the medium to maintain the constant pH. This process caused more HCO<sub>3</sub><sup>-</sup> being formed to maintain a constant ratio of carbonic acid and bicarbonate under pH 6.5. According to pH calculation and the recipe of Bristol medium, the amount of CO<sub>3</sub><sup>2-</sup>, OH<sup>-</sup>, H<sup>+</sup> and [MgOH<sup>+</sup>] are negligible. Therefore, bicarbonate is considered as the major resource contributing to alkalinity at pH 6.5 as can be seen in **Eq. 5-1**.

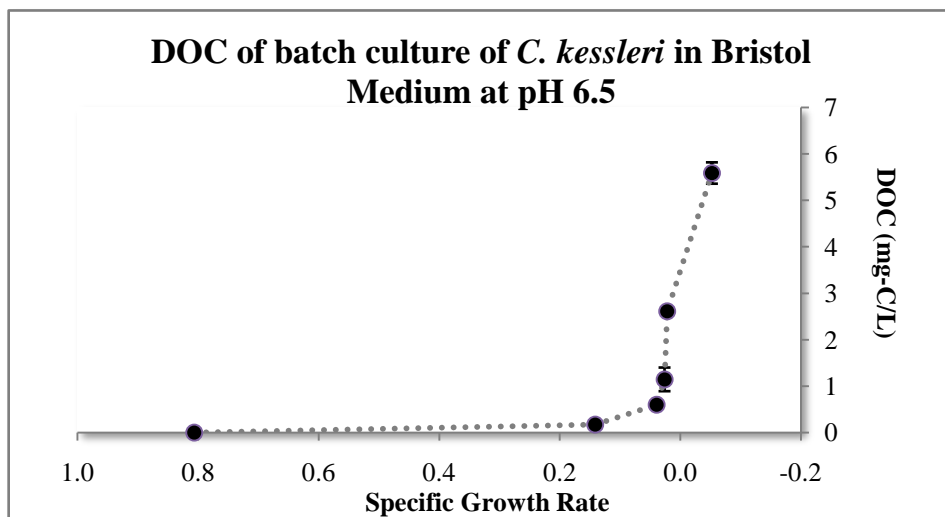
$$\text{TA} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{OH}^-] + [\text{MgOH}^+] - [\text{H}^+] \text{ (Eq. 5-1)}$$

As shown in **Figure 5-14** and **5-15**, the variation of the total alkalinity with the specific growth rate showed a very similar trend as the variation of DOC. The specific growth rate of *C. kessleri* in the Bristol medium decreased with the increase of biomass concentration from the exponential phase to the stationary phase. The total alkalinity and

DOC had a slight increase in the early exponential phase when the specific growth rate was high. Then both of them increased dramatically when the specific growth rate turned to be lower than  $0.2 \text{ d}^{-1}$ .



**Figure 5-14.**Total alkalinity of Bristol medium varied with the specific growth rate of *C. kessleri* at pH 6.5. Error bars stands for  $2 \times \text{SD}$  (standard deviation).



**Figure 5-15.**Dissolved organic carbon concentration of Bristol medium varied with the specific growth rate of *C. kessleri* at pH 6.5. Error bars stands for  $2 \times \text{SD}$  (standard deviation).

DOC in the batch reactor derived from two sources, the degradation from dead algae and the release from healthy algal cells. Generally, robust algae in the late exponential phase are able to synthesize and release more dissolved organic carbon per unit biomass than the algae in other physiological phases (Watt, 1966). Algal-produced DOC, possibly containing glycolic acid, polysaccharides and various amino acids and sugars, is biologically-available. More than 60% of the algae-produced extracellular organic matter in the early stage of growth can be degraded by algae within 5 days (Nguyen et al., 2005). This type of DOC serves as a carbon resource so that the biomass of *C. kessleri* has a positive correlation with a low concentration DOC.

However, as the biomass concentration increases and growth rate slows down, the DOC released extracellularly by algae turns from lower-weight organic matter to greater-weight polysaccharides (Nguyen et al., 2005). This type of DOC with higher molar weight becomes dominant at the early stage of growth and can limit light penetration, and therefore photosynthetic activity. In addition, concentrated DOC might be capable of reacting with iron and phosphorus to form chemical-stable compounds, thus limiting the availability of nutrient for algae to uptake.

## **6 CONCLUSIONS**

Microalgae as one of the biofuel feedstocks has a great potential to provide a sustainable alternative to fossil fuels. The maximum biomass and biofuel productivity can

be achieved if the relationship of microalgal biochemical composition, nutrient concentration and physiological state is found out. This research focused on comprehensively investigating this relationship by studying the variation of biomass and macromolecular content of 144 L batch-cultured *C. kessleri* and *A. falcatus* along with growth time in different cultivation conditions. These different cultivation parameters include pH, nutrient composition, medium type and algal species. Five major conclusions were obtained from this study:

(1) Due to the deficient inorganic carbon resource in alkali medium, 9.8 times more biomass of *C. kessleri* was found at pH 6.5 than that at pH 8.5. However, there was no significant change in lipid content in Bristol medium with different pH.

(2) Both *C. kessleri* and *A. falcatus* showed higher lipid content, lower protein content and lower biomass yield under N-limited condition (N:P=2:1) than under N-sufficient condition (N:P = 50:1). However, *C. kessleri* showed an outstanding capability of lipid accumulation with lipid content varying from 20% in N-sufficient medium to 54% in N-limited medium. The deficiency of nitrogen in WC medium also promoted phosphorus removal to 71% compared with 39% in N-limited medium. On the other hand, high N:P removal ratio indicates an excessive uptake of nitrogen by algae in N-sufficient medium. Excessive storage of mineral compounds was found in both species in the stationary phase of all cultivation conditions.

(3) Compared with WC medium, Bristol medium has 29 times more concentrated nitrogen and phosphorus, which contributes to a 4 times longer exponential growth period and 1.3 times greater maximum biomass concentration of *C. kessleri*. Due to the severe deficiency of nitrogen, the protein content in the stationary phase in WC medium was only 17% of that in Bristol medium, while the lipid content in WC medium was 2.2 times of that in Bristol medium. The growth limiting factor in Bristol medium with N:P= 2:1 might be the lack of minor nutrient, self-shading effect or DOC accumulation.

(4) The fraction of lipids, carbohydrates and protein were similar for both *C. kessleri* and *A. falcatus* in the N-sufficient WC medium. However, protein and lipid showed a strong response to nitrogen limitation in the stationary phase with dramatic decrease in protein content and increase in lipid content. Compared with *A. falcatus*, *C. kessleri* performs better in biomass accumulation and lipid yield in WC medium with or without sufficient nitrogen.

(5) Lab results demonstrated that there is a strong correlation between bicarbonate concentration and total alkalinity in pH 6.5 medium. This is because  $\text{HCO}_3^-$  was the major ion that contributes to total alkalinity at this pH. Algal growth has a positive correlation with low concentration of DOC. The DOC increased exponentially when specific growth rate was lower than  $0.2 \text{ d}^{-1}$  and achieved 5.5 mg-C/L in maximum when algal growth reaches stationary phase.



In all cultivation conditions investigated in this experiment, the maximum algal biomass was achieved by *C. kessleri* in the stationary phase fed with Bristol medium at N:P= 2:1 and pH = 6.5. The highest lipid yield was obtained by *C. kessleri* cultivated in Bristol medium with N:P = 2:1 and pH 6.5. *C. kessleri* performs better in both algal growth and lipid yield than *A. falcatus*. Future field-scale biofuel production may use the conclusions from this large-scale lab experiment as a guidance to achieve maximum biomass and lipid yield.

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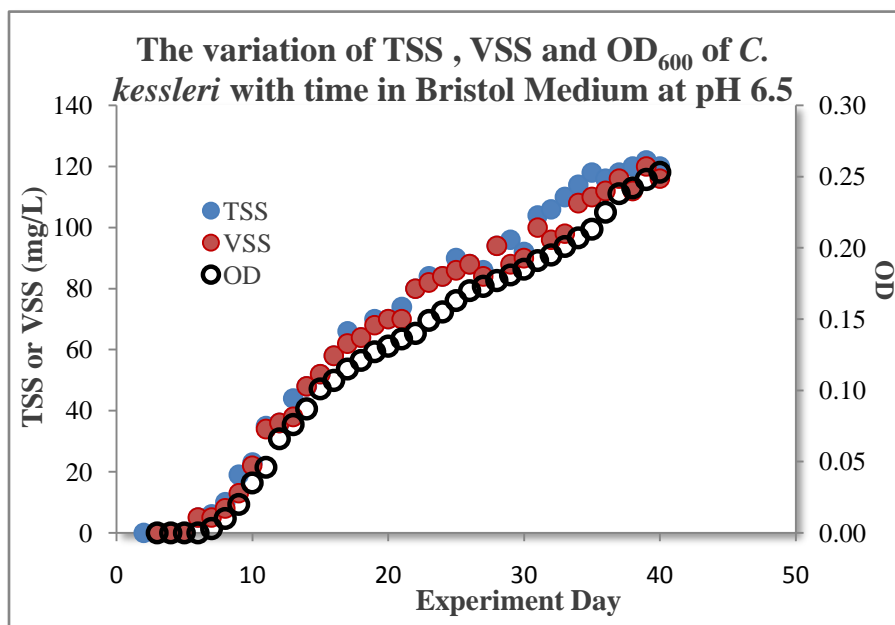
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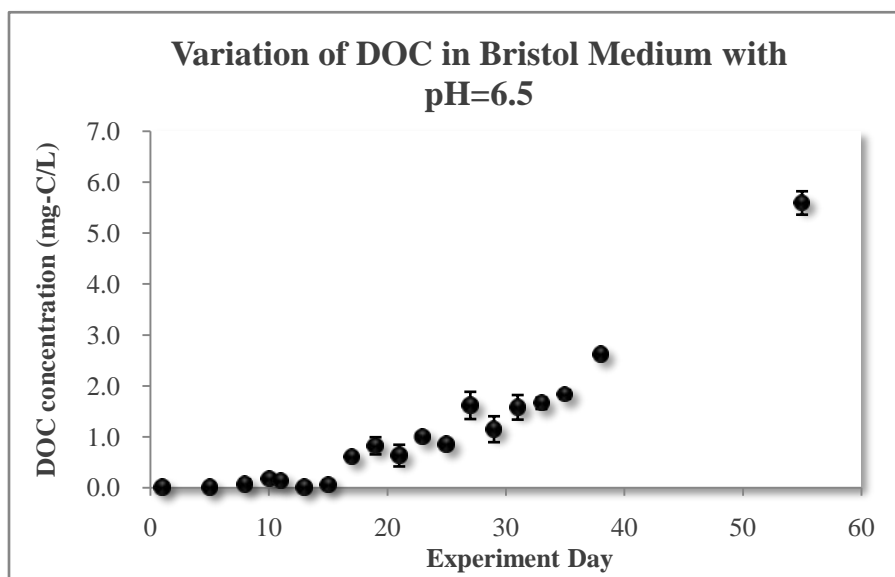
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## APPENDIX A



**Figure 0-1.**Variation of TSS, VSS and OD<sub>600</sub> of *C. kessleri* with time in Bristol medium at pH 6.5



**Figure 0-2.**Variation of DOC in Bristol medium grown with *C. kessleri* at pH 6.5

## APPENDIX B

### Standard Operation Procedure of Protein Assay (Lysis Buffer Method)

#### 1. Chemicals and Solutions

- **Lysis buffer:** 5 ml/L of Triton X-100, 0.3722 g/L of ethylenediaminetetraacetic acid disodium salt, 0.0348 g/L of phenyl methyl sulfonyl fluoride.
- **SDS solution:** 0.05 g/L of sodium dodecyl sulfate salt. 10-fold concentrated stock solution could be made for further use.
- **Bovine Serum Albumin (BSA) solution:** 1 mg (BSA)/mL in 0.15 M NaCl, Sigma Protein Standard Micro Standard, Liquid.
- **Reagent A:** 4.0 g/L of sodium hydroxide and 20.0 g/L of sodium carbonate.
- **Reagent B1:** 0.001 g/L of copper II sulfate pentahydrate. 100 times concentrated stock is suggested to make since weighing such tiny amount of chemicals is very difficult and not accurate.
- **Reagent B2:** 0.002 g/L of potassium sodium tartrate tetrahydrate. 100 times concentrated stock solution could be made.
- **7. Reagent C:** Made up by A, B1 and B2 in a volumetric ratio 100:1:1 (prepared just prior to use).
- **8. Folin-Ciocalteu reagent:** 1:1 v/v Folin reagent / distilled water (prepared just prior to use). Making Folin reagent should be done in the dark or dim-light condition since Folin is light-sensitive.

Since several ingredients that need to be added are in very small quantity, it is better to make the lysis buffer at least 1 L. The stocks of SDS, reagent A, B1 and B2 are suggested to make 500 ml. These solutions would be enough for about 80 measurements. All solutions should be stored in the fridge.

#### 2. Protein Extraction (Pretreatment)

1. Choose a clean and medium size mortar and pestle for milling the freeze-dried algal biomass.
2. Use mortar as a container and measure 20 mg freeze-dried algae powder with an accurate balance (at least 4 decimals).
3. Mill the algal powder with the pestle for 5 minutes.
4. After milling, add 1 ml of lysis buffer into the mortar with a digital pipette to dissolve all the solid biomass. Then add 3 ml of lysis buffer and mix it well. Transfer this 4 ml mixture to a 15 ml Falcon tube. Rinse the mortar and pestle twice with 3 ml lysis buffer each time. All the mixture need to transfer to the Falcon tube and the total volume is 10 ml.

- Place all the Falcon tubes on a shaker table and shake for 1 hr. After that, the mixture suspension should be stored in the fridge. All the tests are better done within 4 hrs. In practical, the macromolecules could be degraded about 2% in 4 hrs.
- Transfer 0.1 ml portion of this well-mixed suspension to 2 ml micro-centrifuge tubes. Label all the tubes with p1, p2 and etc.

### 3. Standards preparation

- At least five standards are needed to be made in this assay. Commonly the concentrations of the standards are 0, 0.2, 0.4, 0.6, 0.8 g/L of BSA (protein standards).
- Place 5 more 2 ml micro-centrifuge tubes for standards and label them as s1, s2, s3, s4 and s5.
- Use 100  $\mu$ l digital pipette pipettes lysis buffer and BSA standards into each tube following the formula below:

Standards ID	S1	S2	S3	S4	S5
Standards Conc. (g/L)	0	0.2	0.4	0.6	0.8
Total Volume ( $\mu$ L)	100	100	100	100	100
BSA Volume ( $\mu$ L)	0	20	40	60	80
Lysis Buffer Volume ( $\mu$ L)	100	80	60	40	20

- Since the maximum concentration of standards is 1 g/L, the samples need to be diluted with lysis buffer if the estimated protein concentration exceeds this value.

### 4. Protein Analysis (Lowry Method)

- Now all the 2 ml centrifuge tubes are placed on a rack, labeled and contained with 0.1 ml of standards or samples.
- Add 0.1 ml of SDS solution to each tube and vortex it.
- Reagent C (1 ml) was added to tubes. Vortex tubes and let them stand for 10 minutes.
- Then 0.1 ml of Folin reagent was added. This was immediately followed by vortex mixing. This process and all the following processes should be conducted in the dark condition since Folin reagent is very light-sensitive.
- After 30 minutes incubation in the dark, centrifuge all the solutions at 3000 rpm for 1 minute.
- Transfer the supernatant to disposable 4.5 ml plastic cuvettes. Measure the absorption at a wavelength of 750 nm in a spectrophotometer. Use the DI water to zero it before reading unknowns.



$$7. \text{ Content of protein} = \frac{\text{Measured protein conc.} \cdot \left(\frac{\text{mg}}{\text{mL}}\right) \times 0.1 \text{ ml} \times 100}{20 \text{ mg}} \times 100\%$$

## 5. Waste Disposal

Since Folin is an irritative chemical, all the solutions in micro-centrifuge tubes and plastic cuvettes should be collected in a plastic bottle, labeled with an EHS Hazardous Material label.

## 6. Reference

Cynthia Victoria Gonzalez Lopez, Maria Del Carmen Ceron Garcia, Francisco Gabriel Acien Fernandez, Cristina Segovia Bustos, Yusuf Chisti, Jose Maria Fernandez Sevilla (2010). Protein Measurements of Microalgal and Cyanobacterial Biomass. *Bioresource Technology*, 101, 7587-7591.

## APPENDIX C

### Standard Operation Procedure of Carbohydrates Assay (Lysis Buffer Method)

#### 1. Chemicals and Solutions

##### 1. 75% H<sub>2</sub>SO<sub>4</sub> solution:

This solution should be made preferably 1 day before the experiment or at least 4 hours before starting. Store the solution in fridge. 500 ml is good for approximately 70 measurements.

- Put 100 ml of DI water in a 500 ml volumetric flask.
- Place a magnetic stirring bar in the flask and put the flask in an ice-bath.
- Wearing goggles, acid-duration thick gloves and lab coat, measure 390 ml of 95%-97% concentrated H<sub>2</sub>SO<sub>4</sub> and add it in flask slowly. This process should be conducted in the fume hood.
- Use more ice to let it cool to room temperature and adjust the volume to 500 ml.

##### 2. Anthron solution:

Prepare anthron solution freshly every time.

- Weigh 0.1 g of anthron powder with a plastic weighing dish.
- Use 2 ml of ethanol to dissolve the anthron and wash it into a 50 ml volumetric flask. 1000 µl of digital pipette can be used for this step. Add ethanol twice and 1 ml for each time to wash it completely.
- Fill 75% H<sub>2</sub>SO<sub>4</sub> to 50 ml slowly.
- Put a magnetic stirring bar in the flask. It will take around 15 minutes to let anthron dissolve.

Every measurement will use 4 ml anthron solution. 50 ml anthron solution is enough for 12 measurements (standards + samples).

##### 3. 20 g/L Glucose standard solution:

This should be prepared freshly. Measure 2.0000 g glucose and transfer to a 100 ml volumetric flask. Fill up to 100 ml with DI water. This measurement should be as accurate as possible.

#### 2. Carbohydrate Extraction (Pretreatment)

1. Choose a clean and medium size mortar and pestle for milling the freeze-dried algal biomass.
2. Use mortar as a container and measure 20 mg freeze-dried algae powder with an accurate balance (at least 4 decimals).
3. Mill the algal powder with the pestle for 5 minutes.
4. After milling, add 1 ml of lysis buffer into the mortar to dissolve all the solid biomass. Then add 3 ml of lysis buffer and mix it well. Transfer this 4 ml mixture

- to a 15 ml Falcon tube. Rinse the mortar and pestle twice with 3 ml lysis buffer each time. All the mixture need to transfer to the Falcon tube and the total volume is 10 ml.
- Place all the Falcon tubes on a shaker table and shake for 1 hr. After that, the mixture suspension should be stored in the fridge. All the tests are better done within 4 hrs. In practical, the macromolecules could be degraded about 2% in 4 hrs.
  - Dilute the samples 10 times. Transfer 100  $\mu$ l portion of this well-mixed suspension and add 900  $\mu$ l of lysis buffer to 15 ml Falcon tubes. Label all the tubes with p1, p2 and etc.

### 3. Standards preparation

- At least five standards are needed to be made in this assay. Commonly the concentrations of the standards are 0, 0.02, 0.04, 0.06, 0.80 g/L of glucose (carbohydrate standards).
- Place 5 more 15 ml Falcon tubes for standards and label them as s1, s2, s3, s4 and s5.
- Use 100  $\mu$ l digital pipette pipettes lysis buffer. Since the volume of 20 g/L standard solution needed is very small, it is suggested to use a 10  $\mu$ l glass syringe to measure this volume to make it more accurate. The standards are made following the formula below:

Standards ID	S1	S2	S3	S4	S5
Standards Conc. (g/L)	0	0.02	0.04	0.06	0.08
Total Volume ( $\mu$ L)	1000	1000	1000	1000	1000
20 g/L glucose standards Volume ( $\mu$ L)	0	1	2	3	4
Lysis Buffer Volume ( $\mu$ L)	1000	999	998	997	996

### 4. Carbohydrates Analysis (Lowry Method)

- Now all the 15 ml Falcon tubes are placed on a rack, labeled and contained with 1 ml of standards or samples.
- Put the rack on ice to chill the samples or standards.
- Add 2 ml of already chilled 75%  $\text{H}_2\text{SO}_4$  to each tube and vortex it.
- Add 4 ml of already chilled anthron solution to the tubes. Cap and vortex to mix.
- Fill half of 300 ml glass beaker with tap water and heat it on the stir plate to boil.
- When the water boils, put Falcon tubes in the beaker and boil for 15 minutes at 100 Deg. C.
- Remove the tubes from hot-water bath. Let it cool to room temperature.

8. Vortex and pour a small portion of the solution in the Falcon tube into disposable 4.5 ml plastic cuvettes. Measure the absorption at a wavelength at 578 nm with a spectrophotometer. Use the DI water to zero it before reading unknowns.

9. 
$$\text{Content of Carbohydrate} = \frac{\text{Measured carbohydrate conc.} \left( \frac{\text{mg}}{\text{mL}} \right) \times 1 \text{ mL} \times 1000}{20 \text{ mg (weight of biomass)}} \times 100\%$$

## 5. Waste Disposal

All the solutions in Falcon tubes and plastic cuvettes should be collected in a plastic bottle since the solution contains high concentration of sulfuric acid. Label the waste bottle with an EHS Hazardous Material label.

## 6. Reference

Cynthia Victoria Gonzalez Lopez, Maria Del Carmen Ceron Garcia, Francisco Gabriel Acien Fernandez, Cristina Segovia Bustos, Yusuf Chisti, Jose Maria Fernandez Sevilla (2010). Protein Measurements of Microalgal and Cyanobacterial Biomass. *Bioresource Technology*, 101, 7587-7591.

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